

**THE EXPRESSION OF AN INSECT ACETYLCHOLINESTERASE IN YEAST**

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## ABSTRACT

One particular mechanism of resistance to organophosphate and carbamate insecticides is insensitive variants of the target enzyme acetylcholinesterase (AChE). The aims were to set up an expression system in *Saccharomyces cerevisiae* for insect AChE with the long term objective of studying mosquito AChE variants and to assess the potential of a yeast system for the large scale production of AChE.

Initially work centred on the analysis of an expression construct pBM150-DACHe containing the *Drosophila melanogaster* AChE (DACHe) cDNA under the yeast *GAL10* promoter. Preliminary analyses were conducted using the assay developed by Ellman *et al* (1961). High levels of interference were encountered on cell breakage making assessments of expression extremely difficult. This interference was found to be due to thiol groups within the yeast cell wall. Enzymatic digestion of the cell wall was found to reduce interference to a manageable level. Analysis of protoplasts indicated that active AChE was either not being expressed or being expressed at undetectable levels. AChE mRNA could not be detected by Northern blotting.

The DACHe cDNA was ligated into the high copy number vector pG3 under the constitutive glyceraldehyde-3-phosphate dehydrogenase gene (*GPD*) promoter to form the construct pG3-DACHe which was transformed into the BJ2168 strain of *S. cerevisiae*. Attempts were also made to subclone the DACHe cDNA into a steroid inducible vector (p2UG) and a secretion vector (pPIC9). Analysis of expression from construct pG3-DACHe by both the Sabine (1955) and Ellman methods revealed that biologically active AChE was being constitutively expressed by *S. cerevisiae*. The expressed DACHe was further authenticated by inhibition with the insecticide Bendiocarb and the inhibitor



phenylmethylsulfonyl fluoride. A  $K_{cat}$  value for the expressed DChE was approximated at 6429.3 molecules  $s^{-1}$  but specific activities were found to be low ( $\approx 0.05$  Units).

The percentage of total *S. cerevisiae* protein that was active DChE was estimated at 0.0009%. SDS-PAGE analysis indicated that this active percentage was likely to be commensurate with the total amount of enzyme protein translated. Northern blotting of total RNA detected low levels of a shortened transcript that may either translate to a truncated but enzymatically active DChE or may represent a transiently stabilised mRNA in the process of degradation. The presence of a small population of full length transcripts that remained undetected should not be discounted. The expressed DChE was found to be located in the cell membrane of *S. cerevisiae* suggesting that it had passed through the secretory pathway and further that the active site was probably internal of the membrane rather than externally situated. Plasmid copy number estimations and growth rate analyses showed that the expressed DChE did not have a deleterious effect on cellular metabolism.

RT-PCR was used to generate a homologous cDNA probe that could in the future be used to screen a cDNA library from *Culex molestus* for a susceptible mosquito AChE gene.

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## DECLARATION

This thesis has not been submitted in whole or in part to this or any other university for any degree and is, except where otherwise stated the original work of the author.

Signature:  .

Date: 30-10-<sup>c</sup>18 .

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## ABBREVIATIONS

Ace	Acetylcholinesterase gene
AChE	Acetylcholinesterase
ACHI	Acetylcholine iodide
AOX1	Alcohol oxidase gene
ARS1	Autonomous replicating sequence (chromosomal)
ATCHI	Acetylthiocholine iodide
BSA	Bovine serum albumin
CEN4	Yeast centromere
CIAP	Calf intestinal alkaline phosphatase
CYC1	Iso-1-cytochrome C gene
DACHe	Acetylcholinesterase from <i>D. melanogaster</i>
dATP	Deoxyadenosine 5'-triphosphate
dCTP	Deoxycytidine 5'-triphosphate
DEPC	Diethylpyrocarbonate (2.32)
dGTP	Deoxyguanosine 5'-triphosphate
DMSO	Dimethylsulfoxide
dNTP	Deoxynucleoside 5'-triphosphate
DTNB	5:5-dithiobis-2-nitrobenzoic acid
DTT	Dithiothreitol
dTTP	Deoxythymidine 5'-triphosphate
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
Endo.R.Stop	Restriction digest stop solution (2.11)
ER	Endoplasmic Reticulum
EtOH	Ethanol
GAL1	Galactokinase gene
GAL10	Galactose epimerase gene
GPD	Glyceraldehyde-3-phosphate dehydrogenase gene
GPI	Glycophosphatidylinositol
GREs	Glucocorticoid response elements
GTE	Glucose Tris EDTA solution (2.8.1)
GTME	RNA extraction buffer (2.32.1)



<i>HIS4</i>	Dominant selectable marker for histidine
IPA	Isopropyl alcohol
IPTG	Isopropylthio- $\beta$ -D-galactoside (2.17)
KOAc	Potassium acetate
L.B.	Luria Bertani Medium (2.3)
mAP	Messenger affinity paper
MOPS	Morpholinopropane sulphonic acid (2.29)
M.M.	Yeast Minimal Medium (2.2)
NaOAc	Sodium acetate
NH <sub>4</sub> OAc	Ammonium acetate
Oligo dT	Oligomer of deoxythymidylate nucleotides
PEG	Polyethylene glycol
PGK	3-phosphoglycerate kinase gene
PMSF	Phenylmethanesulfonyl fluoride
RNase	Ribonuclease
RT-PCR	Reverse transcription PCR
SAM	S-adenosylmethionine
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis (2.24)
SDW	Sterile distilled water
SHP	Snail Helix Pomatia Juice
SSC	Sodium chloride:sodium citrate solution (2.18)
SZB	Protoplasting solution (2.9)
TAE	Tris acetate EDTA buffer (2.12)
Taq	DNA polymerase from <i>Thermus aquaticus</i>
TBE	Tris borate EDTA buffer (2.32.2)
TCA	Trichloroacetic acid
TE	Tris EDTA buffer (2.8.1)
TEMED	Tetramethylethylenediamine
TPB	Triton phosphate buffer (3.2)
Tris	Tris(hydroxymethyl)aminomethane hydrochloride
<i>TRP1</i>	Dominant selectable marker for tryptophan
2 $\mu$	Yeast 2- $\mu$ m circle origin of replication
<i>URA3</i>	Dominant selectable marker for uracil

X-Gal	5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (2.17)
YCp	Yeast centromeric plasmid
YEp	Yeast episomal plasmid
YIp	Yeast integrative plasmid
YPD	Yeast rich medium (2.7)

## CHAPTER ONE INTRODUCTION

The successful functional expression of the essential neurological enzyme acetylcholinesterase (AChE) has been achieved in heterologous systems. The enzyme from *Torpedo californica* (electric ray) in mammalian cells (Gibney and Taylor, 1990; Gibney et al, 1990), the human enzyme in *Xenopus* oocytes (Soreq et al, 1990;), in mammalian cells (Velan et al, 1991a) and also in *Escherichia coli* (Fischer et al, 1993), and that of *Drosophila melanogaster* by microinjection of *Xenopus* oocytes (Fournier et al, 1992; Mutero and Fournier, 1992). AChE from *T. californica* and *D. melanogaster* and from the mosquito *Ae. aegypti* has also been successfully expressed in baculovirus systems (Radic et al, 1992; Chaabihi et al, 1994; Anthony et al, 1995). Very recently, vertebrate AChE from rat and also snake venom has been expressed using the commercially available *Pichia pastoris* yeast system (Morel and Massoulie, 1997).

In addition to being cheap and convenient, heterologous expression in yeast offers the powerful possibility of using *in vivo* oligonucleotide mutagenesis to generate families of variant proteins for isolation and analysis with respect to AChE activity and inhibitor sensitivity.

An assessment of the potential of a yeast expression system for large scale industrial production is also worthy of investigation. The vast majority of commercially available AChE is currently extracted from animal tissues. The development of a suitable heterologous production system is therefore highly desirable because it could supply unlimited quantities of AChE from any source, for which a cDNA is available, at minimal cost. *Saccharomyces cerevisiae* offers a low cost eukaryotic microbial system and unlike *Pichia pastoris* has a long history of safe use in large scale fermentation processes. Studies on the structure, function, and expression of AChE are the focus



for research programmes concerned not only with the problem of insecticide resistance due to insensitive AChE variants in vectors of disease and agricultural pests, but also for research into neurodegenerative disorders such as Alzheimer's and Parkinson's disease (Zang and Misra, 1993; Bogdanovic et al, 1993; Lahiri and Farlow, 1994; Lowenstein et al, 1993), and investigations into AChE's potential as a therapeutic in cases of organophosphate poisoning or as a possible prophylactic or antidote for military 'nerve gases' (Ashani et al, 1991; Raveh et al, 1989). *S. cerevisiae's* long history of safe use in the fermentation industry makes it a particularly suitable recombinant expression system for a product with therapeutic potential. A source of large, cheap, and safe quantities of the enzyme may have considerable commercial possibilities.

### 1.1. Acetylcholinesterase and the Problem of Insecticide Resistance.

The effective control of mosquito borne diseases such as malaria depends in part upon the use of insecticides to limit transmission. Two major groups of insecticides, the carbamates and the organophosphates, act as competitive substrates for AChE (O'Brien, 1976). One mechanism of resistance to these insecticides is due to the existence of insensitive enzyme variants (Oppenoorth, 1985). In mosquitoes, insensitive AChE has been reported in the *Culex pipiens* complex (Raymond et al, 1986; Tang et al, 1990; Severini et al, 1993; Rivet et al, 1994) and in several species of *Anopheles*, including *Anopheles albimanus*, the most important vector of malaria in Central America and northern South America (Ayad and Georghiou, 1975; French-Constant and Bonning, 1989).

The work to date on *D. melanogaster* insensitive AChE variants has demonstrated the importance of the examination of theoretical data in conjunction with sequence data

obtained from field resistant populations. Experimental combinations of mutations can be tested in heterologous systems and possible mutations investigated that are so far unreported from the field. In this way information can be gathered concerning the future potential of AChE genes that confer resistance, whether that potential is inexhaustible or fairly limited. Theoretically there should be a balance between resistance to competitive inhibition by insecticide and the ability of the mutant enzyme to perform its essential role in the nervous system.

The usefulness of *D. melanogaster* as a model of insecticide resistance is not in doubt. However, this well characterised fruit fly is of little importance as a pest species and is not targeted by insecticide treatment as such. Indeed, resistant populations of *Drosophila* have evolved probably only because of accidental exposure (Pralavorio and Fournier, 1992). In contrast diseases transmitted by mosquitoes remain some of the most important health problems in the world today and insecticide campaigns form an important arm of disease control strategies. It has been suggested that if mosquitoes are to be manipulated in an effective way they must become as well characterised as *D. melanogaster* (Crampton et al, 1990). It is important, therefore, to investigate resistance due to insensitive AChE at the molecular level in insects of medical or agricultural importance such as mosquitoes. Variants of an originally susceptible mosquito AChE gene from a species such as the British *Cx. molestus* could be examined in a heterologous system, in the same manner as *D. melanogaster*, for mutations or combinations of mutations conferring resistance, that have been implicated either from sequence data from resistant *Cx. pipiens*, or theoretically from the 3D *Torpedo* structure which recently became available (Sussman et al, 1991). *Cx. molestus* is part of the *Cx. pipiens* species complex which also includes *Cx. pipiens*, a vector of Rift Valley fever in the Nile



delta, and the important vector of lymphatic filariasis, *Cx. quinquefasciatus* (White, 1982). *Cx. molestus* is often described as *Cx. pipiens* (form *molestus*) or as an infrasubspecies of *Cx. pipiens* since the two are virtually inseparable except in terms of ecology and behaviour and very fine details of morphology (Marshall, 1938; Knight and Stone, 1977). It would be interesting to examine whether two AChEs, one fulfilling the known neurophysiological role and the other of unknown function exist in *Cx. molestus* as has been recently suggested for *Cx. pipiens* (Bourguet et al, 1996). Although, investigations of other species of mosquito including *Cx. hortensis* have shown the existence of only one AChE encoded at the Ace locus as is the case in *D. melanogaster* and other insects (Bourguet et al, 1997). Anthony et al (1995) reporting on the cloning and functional expression of the AChE gene from *Ae. aegypti* in a baculovirus system stated the intention of manipulating the *Aedes* gene in order to study resistance conferring mutations. They have now examined by site-directed mutagenesis mutations that are known to confer resistance in *D. melanogaster* and found that they also confer resistance to organophosphate and carbamate insecticides on the *Ae. aegypti* AChE (Vaughan et al, 1997). *Ae. aegypti*, the yellow fever mosquito, is an important disease vector, but could still be considered a model, albeit a mosquito model, in view of the fact that resistance due to insensitive AChE has yet to be reported from a naturally occurring resistant population of *Ae. aegypti*. In contrast, insensitive AChE as a mechanism, has been widely reported from field populations of the *Cx. pipiens* complex (Khayrandish and Wood, 1993a; 1993b; Rodriguez, 1993; Cheikh and Pasteur, 1993; Rivet et al, 1994).

In the light of the current paucity of knowledge regarding the mutations occurring within the insensitive AChEs of mosquitoes, in addition to the important assessment of the potential of a *S. cerevisiae* based system for the large



scale production of AChE, the project was also concerned with the setting up of a yeast system that would be suitable for the expression of a susceptible AChE from the mosquito *Cx. molestus* with the long term aim of studying insecticide resistant variants. Necessarily, investigations with the goal of better understanding enzyme function at the molecular level by the use of heterologous expression systems begin with an appreciation of enzyme structure.

## 1.2. The Structure of AChE

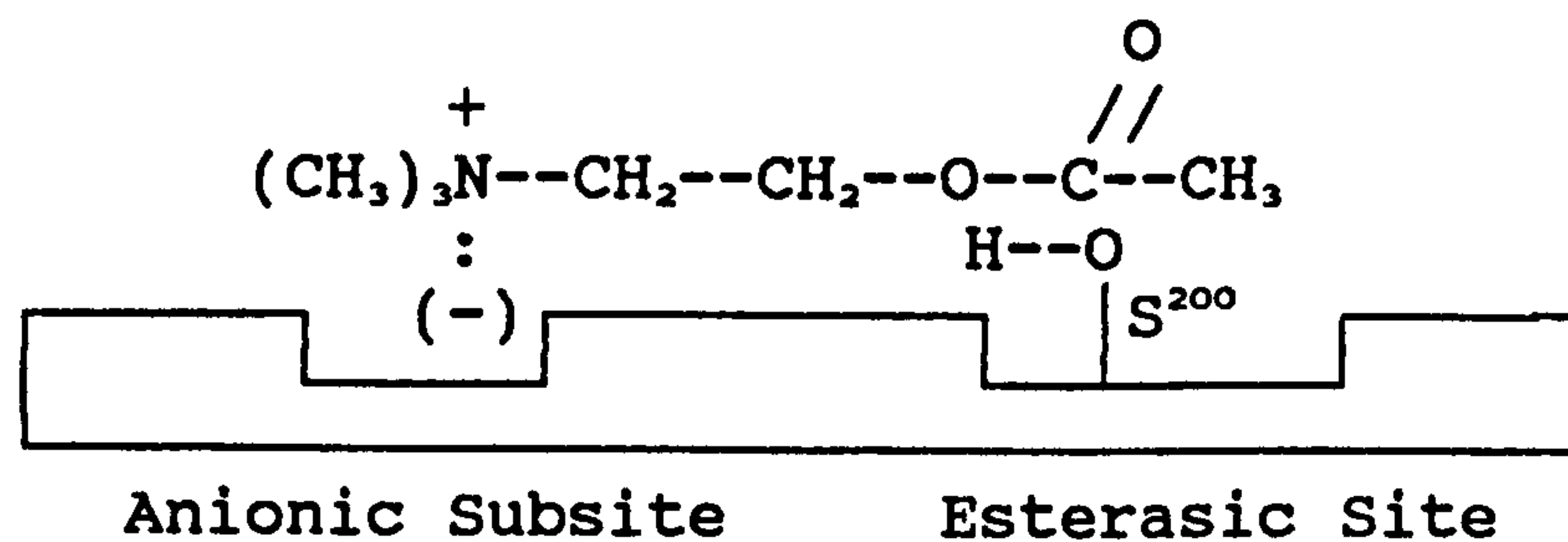
Cholinesterases may be split, for the purposes of classification, into two groups: the acetylcholinesterases (AChEs) and the butyrylcholinesterases (BuChEs) (Taylor, 1991). Both groups belong to a unique family of serine hydrolases that share a sequence similarity confined to four amino acid residues around the active site. This active site, centred on serine-200, involves a putative 'catalytic triad' which also includes a histidine residue at about position 440 and probably a glutamate or aspartate residue (Taylor, 1991; Soreq et al, 1992).

AChE and BuChE have specificities for acetylcholine and butyrylcholine respectively and hydrolysis occurs through electron transfer within the catalytic triad (Soreq et al, 1992). The serine residue, having been activated by the loss of an electron to histidine, is able to react with the substrate, resulting in the formation of a covalent bond as the hydroxyl group of the activated serine is acylated. Subsequent hydrolysis of the bond restores the catalytic serine to the non-active state and acetate or butyrate is released (Sussman et al, 1991; Soreq et al, 1992; Taylor, 1991) (Figure 1.1).

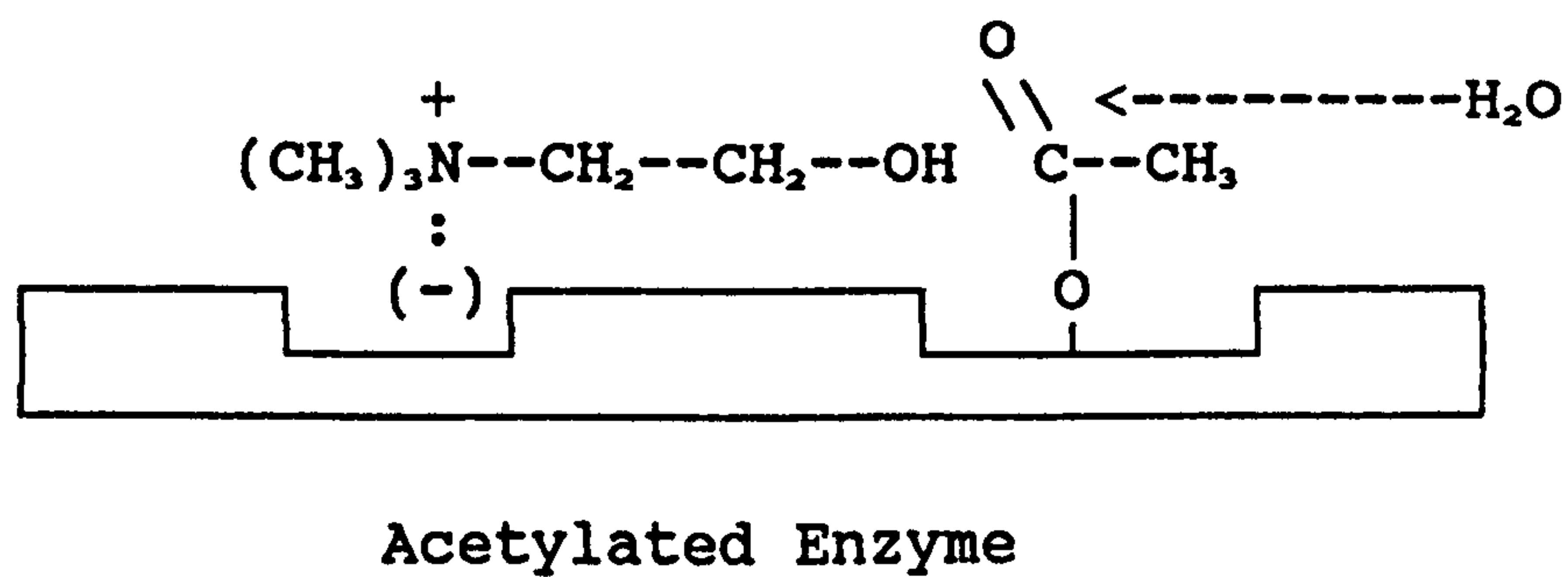
In vertebrates both cholinesterases are found. The physiological role of AChE within the cholinergic nervous

Figure 1.1. Schematic representation of the hydrolysis of acetylcholine by acetylcholinesterase. The esterase site contains the catalytic triad. The hydroxyl group of Serine 200 is acylated whilst the choline moiety is held in a postulated anionic subsite by electrostatic attraction (Stage 1). Hydrolysis of the covalent bond formed with Serine 200 (Stage 2) release the products and restores the enzyme to its non-interactive state (Stage 3) (after Cremlyn, 1978).

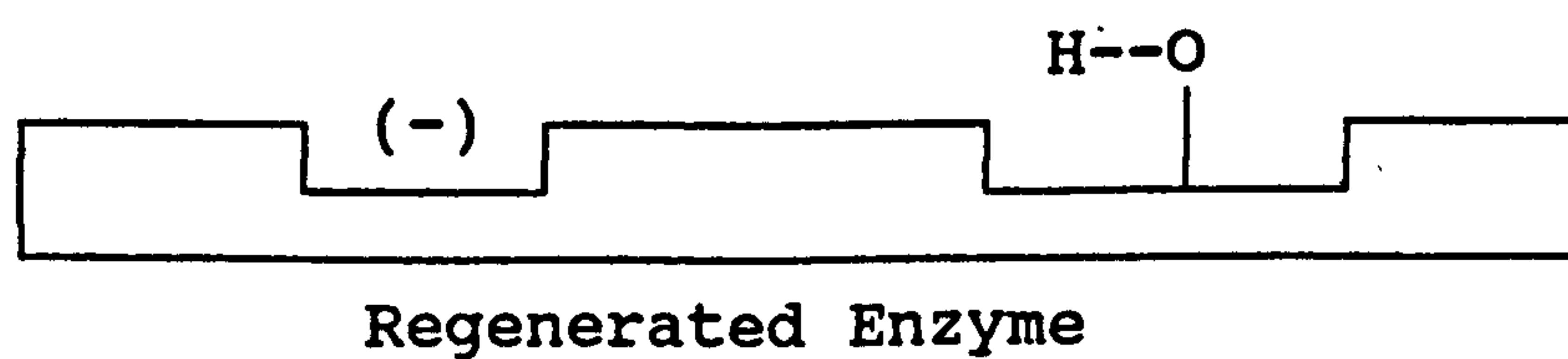
Stage 1



Stage 2



Stage 3



+

$$\begin{array}{c}
 + \\
 (\text{CH}_3)_3\text{N} \text{---} \text{CH}_2 \text{---} \text{CH}_2 \text{---} \text{OH} \\
 \text{CHOLINE}
 \end{array}$$

AND

$$\begin{array}{c}
 \text{CH}_3 \text{---} \text{CO}_2\text{H} \\
 \text{ACETIC ACID}
 \end{array}$$

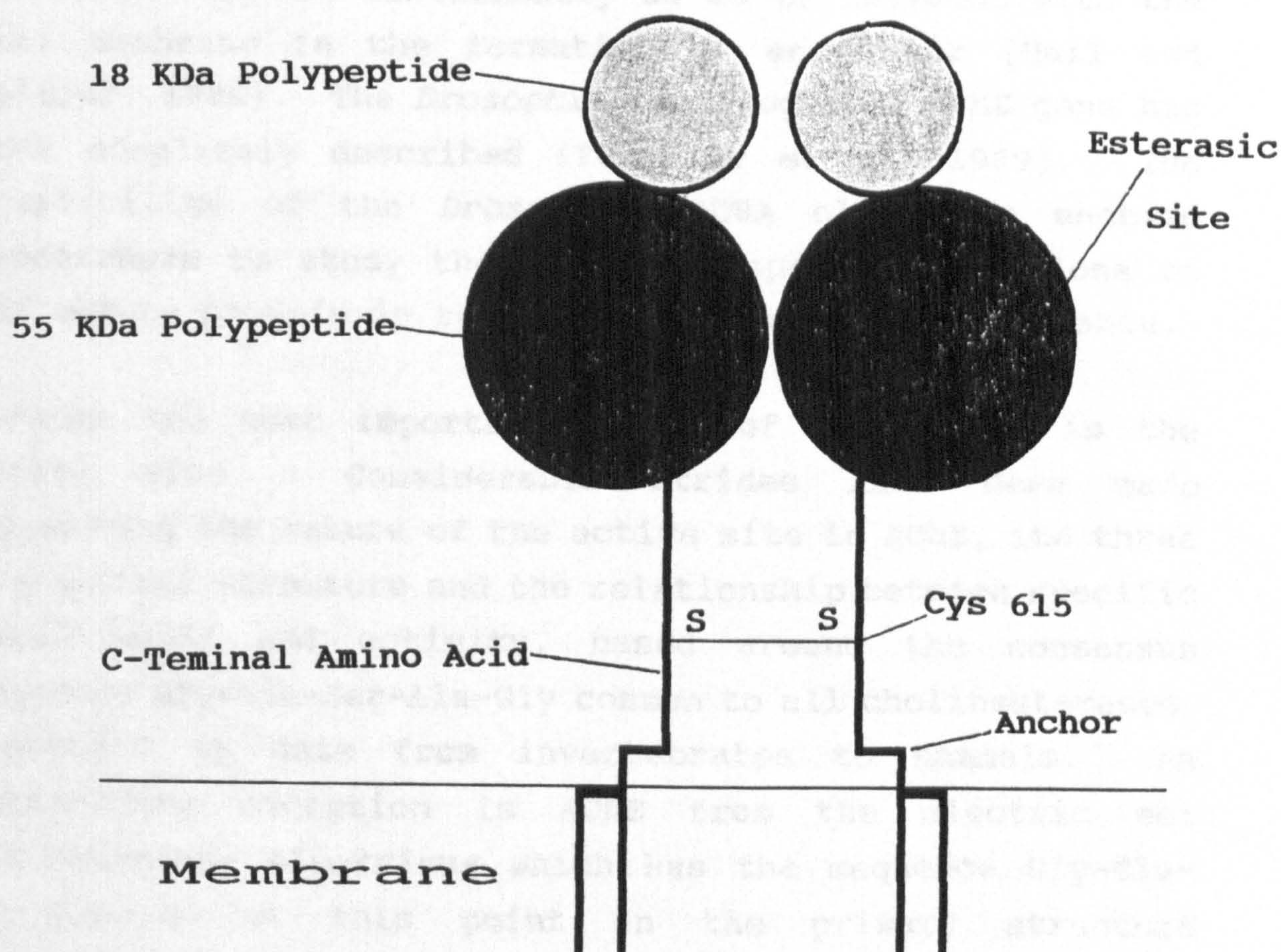


system is well documented, the enzyme being responsible for the termination of neurotransmission mediated by acetylcholine at the synaptic cleft. However, the function of BuChE is less clearly understood. Some workers have suggested that it has a role to play in the detoxification of plant esters that are accumulated from the diet (Soreq and Zakut, 1990). In insects only one cholinesterase has been detected: AChE (Hall and Malcolm, 1991; Mutero et al, 1992).

Much of the work on the molecular structure of AChE has been done on the *Torpedo* enzyme, either from *Torpedo californica* or *Torpedo marmorata*. This is due to the ease with which it can be isolated from this abundant source. Two molecular forms of AChE are described from *Torpedo* and other vertebrates, one of which exists in an asymmetric form comprising two or more usually three catalytic subunits linked to structural subunits and the other as a distinctly globular species that possesses a hydrophobic region (Bon and Massoulie, 1980; Lee et al, 1982; Massoulie and Bon, 1982). In insects only the latter form has been convincingly demonstrated (Toutant et al, 1988) and appears to exist primarily as a dimer attached to the cell membrane by a glycopospholipid tail. It should be mentioned that the two forms of *Cx. pipiens* AChE 1 and 2 that differ with respect to inhibitor sensitivity both exist in this typical insect form (Bourguet et al, 1996). The dimer has been isolated and characterised for *Drosophila melanogaster* (Gnagey et al, 1987) each monomer consisting of a 55 KDa polypeptide (containing the active site) non-covalently associated with a smaller 18 KDa chain and bound to the membrane by the glycopospholipid anchor (Figure 1.2). Fournier et al (1988) provided evidence for the nature of the membrane anchor by successfully converting the hydrophobic enzyme into a soluble form with a specific phospholipase. Furthermore, the first forty amino acids, which form one of the hydrophobic regions of *Drosophila*

Figure 1.2. A model of the *D. melanogaster* AChE dimer (after Fournier et al, 1988). Each active unit is composed of two non-covalently associated polypeptides covalently linked into a dimer at Cys<sup>615</sup> near the C-terminus (Mutero and Fournier, 1992).







AChE (DACE), are not part of the mature protein but make up the signal peptide of 'pre-acetylcholinesterase' as described by Hall and Spierer (1986) which is required for transport across the ER membrane but subsequently cleaved off. The two other hydrophobic regions (near the carboxyl-terminal) are not sufficiently so as to interact with the cell membrane in the formation of an anchor (Hall and Spierer, 1986). The *Drosophila melanogaster* AChE gene has been completely described (Fournier et al, 1989). The availability of the *Drosophila* cDNA clone has enabled researchers to study the effect of specific mutations on the mature protein in relation to insecticide resistance.

Perhaps the most important aspect of any enzyme is the active site. Considerable strides have been made concerning the nature of the active site in AChE, its three dimensional structure and the relationship between specific amino acids and activity, based around the consensus sequence Gly-Glu-Ser-Ala-Gly common to all cholinesterases sequenced to date from invertebrates to mammals. An interesting exception is AChE from the electric eel *Electrophorus electricus* which has the sequence Gly-Glu-Ser-Ser-Glu at this point in the primary structure (Schaffer et al, 1973). When approximately 30% of the primary sequence of venom AChE from the cobra *Naja naja oxiana* was sequenced it too was found to include the predicted consensus sequence around the active site serine (Weise et al, 1990). Studies concerning the interrelation between specific amino acid residues that make up the active site and enzyme activity are dependent upon expression, as is research into the effect that targeted changes make upon resistance to inhibitors. Protein crystallography can map an enzyme and its active site in three dimensions, which may provide inspiration for predicting the importance of certain amino acids which can then be tested experimentally by site-directed mutagenesis. Ogert et al (1990) studied the topography of the catalytic

site of *Torpedo californica* AChE using polyclonal and monoclonal antibody techniques. It was discovered that the region of the enzyme surrounding the active site serine was located in a pocket rather than on the surface and thus inaccessible to antibodies directed against the native conformation. As expected, when the enzyme was reduced and subsequently denatured a high degree of cross-reactivity was observed.

The three-dimensional structure of *Torpedo* AChE has been determined from the crystallized homodimer following purification and enzymatic removal of the anchor. Sussman et al (1991) describe the general structure of the molecule as belonging to the class of alpha/beta proteins and consisting of a twelve stranded central mixed beta sheet surrounded by fourteen alpha helices. The only interchain disulphide bridge of the homodimer is at the carboxyl-terminal end of the molecule and two further bridges are described between two of the longer alpha helices. Also described is an 'active site gorge' penetrating halfway into the molecule, initially narrow but subsequently widening to form the active site. The gorge is approximately 20 angstroms long and serine 200 is located about 4 angstroms above the base. Histidine 440 and, unusually a glutamate residue rather than an aspartate residue at position 327, make up the planar array of the catalytic triad. The structure of the gorge has important implications for the binding of substrates since as much as 40% of the surface was shown to consist of hydrophobic residues. These residues are highly conserved, suggesting an important role in enzyme-substrate interactions, and draw attention to the limitations of making predictions from primary sequence data; for example, residues as far apart as position 66 and 444 are involved (Sussman et al, 1991). It has been suggested that the positively charged quaternary ammonium ion of the choline moiety of acetylcholine may not in fact be bound by an anionic



subsite (as previously thought) but by certain aromatic residues near to the esteric site. Tryptophan 84 in particular is implicated. Thus, an 'aromatic guidance' mechanism rather than an electrostatic mechanism has been postulated for the binding of acetylcholine to AChE. Soreq et al (1992) have suggested that an anionic subsite consisting of several dispersed residues adjacent to the rim of the active site gorge as revealed by the structural data, may in fact be present, and cite an aspartate residue (Asp<sup>70</sup>) situated at the entrance to the gorge as a possible component. This diffuse subsite around the entrance to the gorge may act as a substrate 'sink' enabling a 'lining up' of substrate molecules prior to aromatic guidance and subsequent cleavage at the active site. The constant 'production line' of substrate provided by such a mechanism may account for the high turnover number ( $K_{cat}$ ) of AChE ( $1.6 \times 10^4$  molecules  $\text{sec}^{-1}$  for AChE from *Electrophorus electricus* (Rosenberry, 1975)) in spite of the buried location of the active site and explain the discrepancy between the biochemically predicted anionic site and the aromatic gorge revealed by the 3D structure (Soreq et al, 1992). Electrostatic calculations have indicated that AChE has a strong electrostatic dipole which is aligned directly along the axis of the active site gorge such that a positively charged molecule such as acetylcholine will be drawn towards and down the aromatic gorge. Further analysis revealed that the dipole occurs by virtue of the overall distribution of charge throughout AChE and is not due simply to the acidic residues within the gorge (Glu<sup>285</sup>, Asp<sup>70</sup>, Glu<sup>199</sup>, and Glu<sup>327</sup>) (Ripoll et al, 1993). It appears that such an electrostatic mechanism may act in concert with aromatic guidance in the catalysis of acetylcholine by AChE. The dipole may be important in the initial attraction and direction of substrate towards the entrance of the active site, and draw it into the hydrophobic gorge, where aromatic groups provide a more sensitive and gentler binding than could be achieved with a preponderance of



acidic residues which may bind the substrate too tightly and delay its arrival at the catalytic site. It is significant that the negatively charged amino acids within the gorge are situated behind rather than in front of the lining aromatic residues (Ripoll et al, 1993).

The mechanism whereby the products acetate and choline are efficiently removed is problematic, since diffusion of choline (which is positively charged) out of the active site is opposed by the negative aspect of the electrostatic dipole at the bottom of the gorge. Accumulation of product at the active site is unlikely to occur in as efficient an enzyme as AChE. A 'back door' mechanism for choline release has been suggested on the basis of a molecular dynamics simulation of AChE in water. A transient opening, large enough to pass a water molecule, was discovered near Trp<sup>84</sup> and Met<sup>83</sup> near the base of the active site and such a conformational change could provide an 'exit' from the active site for the choline product (Gilson et al, 1994). Trp<sup>84</sup> is thought to be involved in the binding of the quaternary ammonium of acetylcholine as has been mentioned and although its indole ring points into the active gorge the residue itself is on the protein surface (Ripoll et al, 1993). However this 'back door' hypothesis for product clearance has recently been challenged by site-directed mutagenesis (1.3).

Experiments designed to test hypotheses based upon structural information and molecular modelling depend upon mutagenesis. It has been suggested that random mutagenesis is the method of choice because the least assumptions are made about residues required for protein function (Ernst and Richman, 1989); but the more the information available concerning the detailed structure of the protein together with modelling of substrate binding the more appealing it is to postulate the involvement of certain amino acids. Site-directed mutagenesis is obviously the method of choice

here. The use of site-directed mutagenesis to study and engineer proteins of interest is now widespread. In this way hypothetical sites of importance within a protein may be examined. For example, where sequence data is available, regions of a protein that have been greatly conserved may be tested in order to assess their role in protein function. The three-dimensional structure, if known, can then be used to see how the domains of an enzyme such as AChE fold around its active site and hypotheses made about residues that appear to be a considerable distance from the active site according to the primary structure but may in fact be much closer in space. Specific identifiable mutations circumvent the problem of multiple mutations occurring within the target gene, which lead to further hypotheses about which ones or combinations are responsible for any observed difference in protein function.

### 1.3. The AChE Gene and Heterologous Expression

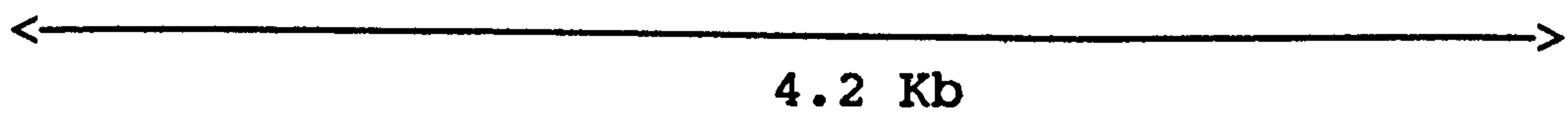
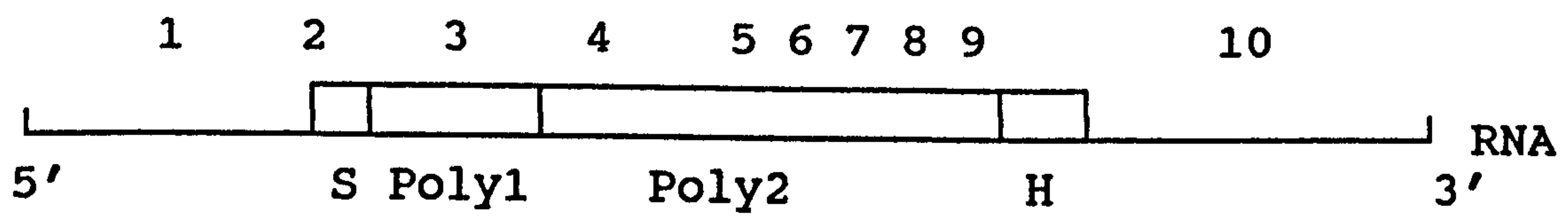
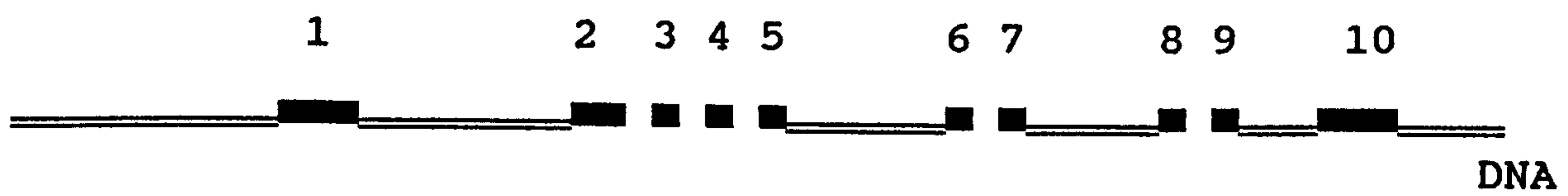
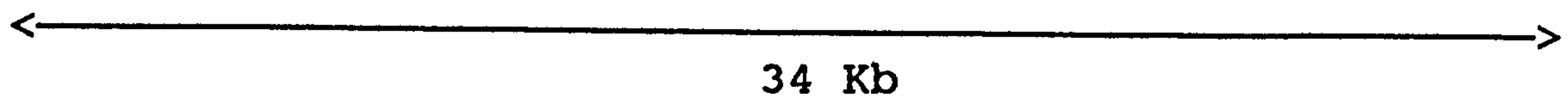
The gene responsible for encoding AChE has been identified and sequenced from *Torpedo*, humans, and several insects. Much of the pioneering work has been done on *Drosophila melanogaster*. The locus (designated *Ace*) was mapped to a region of the third chromosome by Hall and Kankel in 1976 and only more recently was the human gene identified by homology to the *Ace* region of *Drosophila* (Soreq et al, 1985). In *Drosophila* the complete organisation of the locus has been described (Hall and Spierer, 1986; Fournier et al, 1989). The mature mRNA after excision of nine introns and splicing is 4291 bases long without polyadenylation. The 5' leader contains multiple initiators of translation upstream of the main open reading frame. This has also very recently been found to be the case in the 5' leader of the *Ae. aegypti* AChE gene (Liu et al, 1998). The ten coding exons are distributed along 34 Kb of primary transcript. Figure 1.3 shows the molecular



map of the *Ace* locus in *Drosophila*. *Ace* from *An. stephensi* also consists of ten coding exons but encompassed within 3 Kb of genomic DNA. The position of introns is almost identical (Hall and Malcolm, 1991; Malcolm and Hall, 1990). A genomic DNA library from *An. stephensi* was probed using a cDNA fragment containing most of the coding region of the *Drosophila* AChE gene (Hall and Malcolm, 1991). A comparison of the deduced protein sequence of *An. stephensi* AChE and that of *Drosophila* revealed a homology of 69% excluding signal peptides. In the last coding exon of the gene there is little homology between the two insects but it is suggested that most of this non-homologous region may be removed prior to attachment of the glycopospholipid anchor (Hall and Malcolm, 1991). In anchored proteins such as these it has been postulated that this hydrophobic tail of about thirty amino acids may be involved in transiently securing the protein in the ER membrane prior to anchor attachment (Cross, 1987; Low, 1987). From the cDNA clone of the *Ae. aegypti* AChE gene isolated by Anthony et al (1995) the predicted amino acid sequence shares 64% identity with AChE from *Drosophila* and 87% identity with AChE from *An. stephensi*. The gene from the mosquitoes *Culex pipiens* or *Culex molestus* has yet to be described, although partial sequence data for exons 2 and 3 of *Ace* from a resistant strain of *Cx. pipiens* became available during the project (Rooker, 1994). It has been suggested that the *Culex* gene contains long introns more analogous to the ones found in *Drosophila* rather than to the short introns of *An. stephensi*. This has made sequencing of the genomic gene a difficult task and the production of a cDNA clone of the *Culex* gene highly desirable (Rooker, 1994). Although tissue specific post-translational modifications of a single gene may be responsible for the two forms of AChE observed in *Cx. pipiens* previously mentioned, there is also the possibility that the two forms are encoded at two separate loci *Ace1* and *Ace2* (Bourguet et al, 1996).



Figure 1.3. Molecular map of the *Ace* locus in *D. melanogaster* (after Fournier *et al*, 1989). The coding exons of the genomic DNA are shown as blocks and their relationship to the mature RNA transcript indicated numerically. Thus exon 1 is a non-coding exon and exon 10 codes for H and is also transcribed into the 3' untranslated region of the transcript. Single lines = 5' and 3' untranslated regions of the transcript. S = signal peptide; Poly1 = polypeptide chain 1; Poly2 = polypeptide chain 2; H = Gene product of the last exon coding for the attachment of the protein to the membrane, a hydrophobic peptide, that is probably exchanged in the mature protein for the glycopospholipid anchor (Fournier *et al*, 1989).



Heterologous expression has enabled workers to examine essential functional residues in AChE by site-directed mutagenesis. Gibney et al (1990) expressed wild type and several site-directed variants of *Torpedo* AChE in a mammalian expression system and assayed for enzyme activity by the method of Ellman et al (1961) which makes use of a thiol derivative of acetylcholine in the following reaction:

AChE

acetylthiocholine -----> thiocholine + acetate

thiocholine + dithiobisnitrobenzoate -----> yellow colour

The yellow anion is 5-thio-2-nitrobenzoic acid and its formation can be measured by absorbance between 400 and 420nm on a spectrophotometer. Mutation of serine-200 to cysteine affirmed the essential role of the former residue in the active site of AChE by diminished catalytic activity. A mutation to valine resulted in no detectable activity at all. Another residue of the catalytic triad was also investigated, successfully assigning the position 440 to the catalytic histidine rather than 425. The mutation of these conserved residues resulted in abolition of activity for position 440 but only a slight reduction was observed when the histidine at 425 was altered. Mutation of glutamate at position 199, adjacent to the active site, resulted in variants displaying reduced activity. Alteration of specific amino acids does not simply affirm or deny a role in catalysis but provides a whole spectrum of data depending on the mutation selected. For example, a mutation of serine-200 to valine abolishes enzyme activity but an alteration to cysteine results in an enzyme of reduced functional capacity. This indicates that the structure of AChE is similar enough to that of a cysteine hydrolase to function as such (Gibney et al, 1990). A further example is another mutation selected by Gibney et al, that of glutamate-199 to a histidine residue.



No activity was detected despite a histidine residue occurring at this position in a related enzyme, Est-6 in *Drosophila*.

The 'back door' hypothesis formulated by researchers of the Silman and Sussman group for product clearance in *Torpedo californica* AChE (Ripoll et al, 1993; Gilson et al, 1994) has been challenged by site-directed mutagenesis. Kronman et al (1994) tested the hypothesis in the human enzyme by substitution of the putative key residues involved. Replacement of Trp<sup>86(84)</sup> by alanine for example was found to greatly decrease the turnover rate of the enzyme for acetylthiocholineiodide (ATCHI) as expected (because Trp<sup>84</sup> is thought to be involved in the binding of the choline moiety) but the rate for the substrate S-3,3-dimethylbutyl thioacetate (TB) was little affected. Replacement of Trp<sup>86(84)</sup>, the 'gate keeper', by alanine was expected to improve the efficiency of the product clearance mechanism (according to the proposed model) with a concomitant increase in catalytic efficiency (for TB) but in fact a slight decrease was observed. Replacement of another key residue Val<sup>132(129)</sup> thought to be involved in the clearance mechanism, by alanine had no effect on catalysis for either of the two substrates tested; suggesting that the 'proposed back door' is 'probably of no functional relevance' to the catalytic activity of AChE. Mutation of Val<sup>132</sup> by lysine was also investigated by Kronman et al (1994) which had been suggested as a significant test by Gilson et al (1994) because the positive group of a lysine residue situated at that position would effectively seal the 'back door' to product clearance. A large decrease in the turnover rate of ATCHI was observed for the mutant enzyme (but only a slight decrease for TB) but this was thought to be due rather to a local effect of the substitution on the important Trp<sup>84</sup> binding residue, than to inhibition of product clearance. Kronman et al (1994) provided further evidence for this by an inhibitor study using edrophonium

(ethyl(m-hydroxyphenyl)-dimethylammonium chloride). The mutant containing the valine<sup>132</sup>:lysine substitution displayed a marked reduction in affinity for an inhibitor known to interact with Trp<sup>84</sup> (Kreienkamp et al, 1991; Barak et al, 1994). Kronman et al (1994) also make the point that sealing of a 'back door' by lysine should have led to accumulation of edrophonium at the active site and decreased rather than increased the inhibition constant. The question of how the positively charged choline product is efficiently removed from the active site of AChE still remains to be elucidated.

Heterologous expression also allows larger mutations to be examined by the process of deletion. It is known that *Torpedo* AChE is encoded in three exons, exons one and two encoding the primary sequence common to both the asymmetric and hydrophobic forms of the enzyme and the third (designated 3A and 3H) specifying the differing carboxyl terminus. Transfection of mammalian cells with cDNAs of both forms, together with mutants with either a deleted second or third exon, has provided evidence that either exon 3A or 3H is responsible for the cellular location of *Torpedo* AChE (Gibney and Taylor, 1990).

A functional precursor of *Drosophila* AChE has been expressed in *Xenopus* oocytes (Fournier et al, 1992). The cDNA was inserted into a suitable expression vector (pKC4) containing the Simian virus 40 promoter and subsequently introduced into oocyte nuclei by microinjection. The resulting protein was found to be enzymatically active and biochemically similar to *Drosophila*-head AChE, but cleavage of the hydrophobic C-terminal peptide did not occur and there was consequently no replacement by the glycopospholipid anchor. Injection of a cDNA minus the coding sequence for the C-terminus resulted in the secretion of a hydrophilic AChE. Mutero and Fournier (1992) have examined the post-translational modifications



of *Drosophila* AChE using the *Xenopus* expression system in conjunction with site-directed mutagenesis. It was found, by mutating two candidate cysteine residues at positions 328 and 615 to valine and arginine respectively, that it is cysteine 615 that forms the disulphide bridge of the mature 150 Kda protein. The mutation at position 615 gave rise to a monomer of 75 KDa as shown by non-denaturing polyacrylamide electrophoresis. It was also shown that the *Drosophila* protein has four sites, rather than the potential five, of glycosylation at asparagine residues and that a hydrophilic sequence from position 148 to 180 is crucial for the successful cleavage of the protein into subunits of 18 and 55 KDa.

#### **1.4. Insecticide Resistant AChEs and Heterologous Expression**

##### **1.4.1. Insensitive AChEs**

The resistance mechanism that has developed due to subtle alterations in the active site of the target enzyme AChE is a powerful one. Such AChEs (labelled 'insensitive AChEs') display markedly reduced susceptibility to inhibition by insecticides and yet are still able to function effectively in the insect nervous system. The presence of insensitive AChEs has been widely observed in insects. Fournier *et al* (1992) have also provided evidence for the possible contribution of an overexpression of AChE in the central nervous system of resistant insects to insecticide tolerance but the major cause of resistance is due to point mutations which produce alleles that display decreased sensitivity to insecticides and often have differing catalytic properties to the wild type enzyme.

##### **1.4.2. Classical Methods**

Insecticides such as malathion and propoxur inhibit AChE by



reacting with the active site of the enzyme in an analogous way to its natural substrate acetylcholine. The enzyme is phosphorylated or carbamylated as opposed to acylated. The dephosphorylating or decarbamylating steps are, however, extremely slow in comparison to deacylation (O'Brien, 1976). The result is competitive inhibition leading to excessive stimulation at the cholinergic synapse due to the accumulation of acetylcholine (O'Brien, 1976).

Insensitive AChE resistant strains from a wide variety of insect species have been reported and include anopheline and culicine mosquitoes, vectors of malaria, filariases, and many arboviruses (Ayad and Giorghiou, 1975; Hemingway and Davidson, 1983; Hemingway and Giorghiou, 1985; Cheikh and Pasteur, 1993; Khayrandish and Wood, 1993a; 1993b), in addition to non-dipteran agricultural pests such as spider mites (*Tetranychus urticae*) (Smissaert, 1964), aphids (*Aphis gossypii*) (Silver et al, 1995), Colorado potato beetles (*Leptinotarsa decemlineata*) (Wierenga and Hollingworth, 1993; Argentine et al, 1994), and cattle ticks (*Boophilus microplus*) (Roulston et al, 1968). Resistant strains of the housefly (*Musca domestica*) have also been studied and insensitive AChE variants reported from them (Devonshire, 1975; Bull and Xu, 1995).

Much of this work has been accomplished using either bioassays, *in vitro* enzyme assays, or both. Bioassays involve the direct testing of insecticides upon adult or larval insects of different ages and can, with the use of synergists, implicate insensitive AChE specifically as a mechanism. Using triphenyl phosphate (TPP: an inhibitor of carboxylesterases) and piperonyl butoxide (PB: a multi-function oxidase inhibitor) Hemingway and Davidson implicated insensitive AChE as a mechanism in resistant strains of *Anopheles atroparvus* (1983). In a resistant strain, lack of synergism between TPP and malathion indicated that the cause of resistance was unlikely to be

due to any carboxylesterase action, and tests performed with fenitrothion and PB did not produce any significant increase in the level of resistant strain mortality either. Thus resistance was not caused by multi-function oxidase activity. Further testing of parental strains and sub-selection with single insecticides revealed a broad spectrum of cross resistance (i.e that strains selected with one insecticide to a higher level of resistance to that insecticide also exhibited a higher level of resistance to other insecticides when compared to the parental strain). This cross resistance between organophosphate and carbamate insecticides also provided evidence for insensitive AChE involvement because these two groups are not closely related chemically.

This broad range of resistance to both organophosphates and carbamates conferred by an insensitive AChE has been reported in several species of arthropod such as cattle ticks (Roulston et al, 1968) or mosquitoes (Hemingway et al, 1986; Bonning and Hemingway, 1991) but often the resistance observed is fairly or even severely restricted. For example, Wierenga and Hollingworth (1993) found that cross-resistance in a resistant strain of Colorado potato beetle was restricted to carbamates and that the strain was as sensitive to organophosphates as the susceptible strain. AChE resistance in a particular strain of *Cx. pipiens* was reported by Tang et al (1990) as being specific for fenthion alone. Another strain of *Cx. pipiens* has been found to display negative cross resistance (Raymond et al, 1986). This strain, resistant to propoxur, from Southern France was found to be three times as sensitive to dichlorvos as a susceptible strain. This observed 'antiresistance' where insensitivity to one inhibitor increases sensitivity to another has implications, at the molecular level, for the rational design of new antiresistant insecticides.



The enzyme can also be studied using *in vitro* assay techniques. The method of choice is the one developed by Ellman et al (1961). The usual method is to obtain a crude homogenate of a number of adult insects or larvae from a susceptible strain and other strains selected for resistance to certain inhibitors and test the homogenate for AChE activity in the presence and absence of inhibitor. In this way Ayad and Georgiou provided evidence for insensitive AChE as a resistance mechanism in *Anopheles albimanus* (1975). This adaptation of the Ellman method measured the amount of residual enzyme activity remaining after the inhibitor was added for susceptible, parental, and selected strains of *An. albimanus*. The rate of inhibition by parathion and propoxur was found to be considerably faster for susceptible homogenates implicating reduced sensitivity of AChE extracted from resistant insects. Interestingly, the rate of acetylthiocholine hydrolysis in the absence of inhibitor was significantly lower in resistant strains indicating an alteration in the active site of the enzyme. Thus the insensitive enzyme appeared to be less efficient at metabolising the natural substrate than its susceptible counterpart, but this is of little importance when compared to the enormous selective pressure of repeated insecticide campaigns carried out in the wild. Indeed, resistance is usually correlated with a decrease in AChE hydrolysis (Roulston et al, 1968; Devonshire, 1975; Tang et al, 1990; Byrne and Devonshire, 1993).

Further evidence for the enormous selective pressure of insecticide campaigns is given by interactions between pesticides and plant resistance mechanisms that also inhibit AChE. For example in the Colorado potato beetle it has been found that AChEs displaying insensitivity to insecticide may conversely be more sensitive to potato glycoalkaloids such as tomatine and  $\alpha$ -chaconine and thus in a sense 'less fit' than their susceptible counterparts



(Wierenga and Hollingworth, 1992; Argentine et al, 1994).

The Ellman assay lends itself well to the monitoring of kinetic reactions and more recently microtitre plate readers have been employed to provide a more comprehensive picture. Sensitivity is such that single insect homogenates can be replicated and many individuals tested at the same time. Furthermore, assays performed in this way are able to highlight the differences between insects that are homozygote and heterozygote for resistant alleles (Moore et al, 1988). In tests on houseflies the assay successfully distinguished between a total of six possible genotypes arising from the crossing of three different strains each homozygous for a separate allele of Ace (one susceptible and two resistant variants). Resistant strains were selected to homozygosity from single pair crosses.

French-Constant and Bonning (1989) employed this rapid microtitre plate test and Ellman method to distinguish between three different AChE genotypes (susceptible, heterozygous and homozygous resistant to propoxur and malaoxon) in the mosquito species, *An. albimanus*, *An. nigerrimus*, and *Cx. pipiens*, noting the ease and convenience of the technique, in addition to its reliability in discriminating between homozygous and heterozygous individuals, and its potential for monitoring resistance in field populations of mosquitoes. Since then the technique has been used to assess frequencies of naturally occurring AChE resistant strains of *Cx. quinquefasciatus* from Tanzania (Khayrandish and Wood, 1993a; 1993b) and Cuba (Rodriguez et al, 1993) and *Cx. pipiens* from Tunisia (Cheikh and Pasteur, 1993) and the Rhone-Alpes region of France (Rivet et al, 1994) in order to formulate advice on current insecticide strategies.

In this way information can be gathered about the genetic basis for AChE insensitivity using classical breeding

techniques, bioassays and biochemical microtitre plate tests. Such studies have shown not only the existence of insensitive AChE as a mechanism of insecticide resistance but that a single gene is responsible (back-cross progeny of hybrids with the susceptible strain segregate into 50% susceptible and 50% displaying the level of resistance associated with the hybrid; biochemical assays using the Ellman method on AChEs extracted from resistant insects typically give rise to linear inhibition curves implicating only one type of enzyme), that it displays codominance (heterozygotes are typically semi-resistant), and that many insensitive alleles of Ace have arisen leading to differences between resistant strains of the same species with regard to tolerance of specific insecticides.

#### 1.4.3. The Role of Heterologous Expression in the Study of Insensitive AChEs

A variant of AChE conferring a degree of resistance to malathion in *Drosophila melanogaster* was identified by Morton and Singh (1982) and subsequently the mutant gene was cloned, sequenced and compared to wild type. It was found that all mutations were silent with the exception of a phenylalanine residue at position 368 which had been replaced by tyrosine (Pralavorio and Fournier, 1992). This phenylalanine residue is highly conserved and it is interesting to note that it is listed as one of the fourteen aromatic residues of the active-site gorge in *Torpedo* AChE (Sussman et al, 1991).

More recently Mutero et al (1994) reporting on the sequencing of AChE genes from three resistant field strains of *D. melanogaster* identified five point mutations linked to insensitivity to the insecticides maloxon, paraoxon, carbaryl, and propoxur. A different resistance pattern was observed for each mutation. One mutation was a further confirmation of the Phe<sup>368(288)</sup> to Tyr substitution previously

reported; the other four were: Phe<sup>115(78)</sup> to Ser, Ileu<sup>199(Va1129)</sup> to Val, Ileu<sup>199(Va1129)</sup> to Thr, and Gly<sup>303(227)</sup> to Ala. Phe<sup>115</sup>, like Phe<sup>368</sup>, is also one of the aromatic residues of the active site gorge, whilst Ileu<sup>199</sup> resides in space close to Trp<sup>121(84)</sup>, and Gly<sup>303</sup> close to the catalytic serine-276(200). Such mutations probably affect AChE sensitivity by local conformational changes that restrict access to the active site by insecticide. Access by the natural substrate is, in many insensitive variants, probably somewhat restricted too, leading to the lower catalytic efficiencies already mentioned, but to far less of a degree. This crucial difference between active site access by acetylcholine and by any specific inhibitor must necessarily be a reflection of differences in chemical structure. Differences in insecticide structure may also provide an explanation for why insects are often resistant to one insecticide but not another.

It is known that an aspartate to glycine substitution at position 70 in human butyrylcholinesterase gives rise to a variant that exhibits reduced affinity for specific inhibitors (Lockridge and La Du, 1978; McGuire et al, 1989). This conserved amino acid corresponds to a tyrosine residue in insects at position 109 and may be a potential site of insecticide resistance since its location in the active site gorge (Sussman et al, 1991) makes it a likely candidate for a peripheral binding site. It is considered that peripheral interactions such as these occur prior to aromatic guidance and subsequent catalysis (Soreq et al, 1992). Mutero et al (1992) reported the effect of mutations at position 109 in *Drosophila* AChE expressed in *Xenopus* oocytes. It was concluded that substitutions at 109 affect all of the subsites involved in catalysis or inhibition. With respect to insecticide resistance it was found that mutations to glycine, aspartate, or lysine gave rise to variants exhibiting a degree of resistance to certain insecticides; not significant enough to confer real



protection but, perhaps in conjunction with other mutations enhancing resistance potential. Indeed, perhaps the major finding on expression in *Xenopus* oocytes of the wild type *Drosophila* gene containing the five point mutations discovered by Mutero et al (1994) both singly and in combination was that combinations of mutations gave rise to higher levels of resistance than single mutations and further that certain combinations lead to higher resistances than others. For example a resistant variant containing valine at position 199, alanine at position 303, and tyrosine at 368 exhibited high levels of resistance to all four insecticides tested. In contrast, a combination of Tyr<sup>368</sup>, Ala<sup>303</sup>, and Ser<sup>115</sup> produced far lower levels of resistance to all four insecticides. A double mutation to Ala<sup>303</sup> and Tyr<sup>368</sup> with position 115 unchanged was found to produce a higher level of resistance, particularly to the two carbamates tested, than the triplet combination. Mutero et al (1994) conclude that: 'high level resistances are more likely to originate from combinations of point mutations responsible for weak resistances than from the appearance of a point mutation giving a strong resistance'.

It seems likely that these high level resistant combinations arise by recombination events rather than by the accumulation of successive mutations (Mutero et al, 1994) since altered AChE as a mechanism of resistance is not observed in all resistant arthropod species. The accumulation of point mutations under insecticide regimes would be expected to occur randomly regardless of species and this is not observed in the field. Interspecific differences with regard to the length and structure of the gene (for example in *D. melanogaster* the coding region of the gene is distributed over 20Kb of genomic DNA, in *An. stephensi* over only 3Kb), and its chromosomal location will affect intracistronic recombination. Reproductive differences between arthropod species would also affect it. Species which reproduce by parthenogenesis, for example,

for much of the life cycle would be subject to far fewer recombination events.

There is also evidence for pre-existing mutations occurring at significant frequencies in natural susceptible populations. Susceptible populations of *D. melanogaster* from different parts of the world have been shown to display a high degree of heterogeneity with regard to AChE sensitivity and this heterogeneity is in reality synonymous with slight levels of resistance (Pralavorio and Fournier, 1992). It is highly likely that recombination events which lead to high levels of resistance are selected for when arthropods are subjected to continual insecticide treatment campaigns and that in such circumstances any fitness cost becomes irrelevant. It should be stressed that the accumulation of random mutations cannot be ruled out as a mechanism but that intracistronic recombination is probably a more important factor.

The heritable dominance of insecticide resistant alleles should also be considered in insect populations, since dominance levels conferred on offspring can differ between resistant strains of the same insect species that display identical inhibition profiles for an insensitive AChE. This has recently been examined in identically resistant strains of *Cx. pipiens* where it was proposed that expression of AChE may be regulated by modifying neighbouring or even distant loci leading to heritable variation with regard to resistance (Bourguet et al, 1997).

### 1.5. Heterologous Expression in Yeast

The advantages of *S. cerevisiae* as a system for heterologous expression include well characterised genetics, biochemistry and physiology, together with safety, ease of culture, and availability of suitable vectors containing selectable markers that can also be



manipulated and cloned in *E. coli*. Yeast, being a eukaryote, also possesses the considerable advantage of compartmentalised post-translational processing and a complex secretory pathway through which heterologous proteins can be directed if required. Post-translational modification occurs with a high level of fidelity in the secretory pathway, for example correct folding of proteins around disulphide bonds, and glycosylation is accurate but the carbohydrate moieties attached are different to those of higher eukaryotic proteins. It was once hoped that this unicellular eukaryote would be able to correctly process the introns from mammalian primary transcripts but it has since been found that to all intents and purposes this is not the case and that a cDNA clone of the gene of interest is required for expression.

The major limitation of any expression system including yeast is that the heterologous production of a particular protein such as AChE from *S. cerevisiae* is still largely a matter of experiment. Optimization and even any level of expression at all varies widely depending on the strain used, the vector employed, the promoter attached, whether intracellular expression is adequate or the secretory pathway necessary. Efficiency of transcription and translation together with variability in mRNA or protein stability are all factors affecting successful expression of heterologous proteins in yeast (1.5.5).

Proteins may be expressed but if activity is required, for example from an enzyme such as AChE, then modifications after harvesting may be essential, such as solubilization and refolding *in vitro*, before any significant level of activity is observed. Hepatitis B vaccine from yeast requires disaggregation and renaturation following intracellular expression and extraction (Wampler *et al*, 1985). The possibility that modifications may be required post-expression is certainly not limited to production in



yeast. For example, when human AChE was recently expressed in *E. coli*, it only became biologically active after solubilization and refolding, and only significantly so following replacement of the unpaired cysteine-580 residue by serine (Fischer et al, 1993). It should be noted however, that following expression of the mutant containing the Cys<sup>580</sup> substitution, solubilization and refolding of the enzyme were still required for it to become active because the *E. coli* human AChE was found to be embedded in inclusion bodies (Fischer et al, 1993; Fischer et al, 1995).

The wide variety of heterologous proteins that have been successfully expressed in *S. cerevisiae*, apart from hepatitis B vaccine, include: human  $\alpha$ -interferon (IFN) (Hitzeman et al, 1981; Tuite et al, 1982; Dobson et al, 1983), human epidermal growth factor (hEGF) (Urdea et al, 1983; Brake et al, 1984), human  $\alpha_1$ -antitrypsin (Cabezon et al, 1984), bacterial cellulase (Skipper et al, 1985), wheat  $\alpha$ -amylase (Rothstein et al, 1987), mouse immunoglobulin (IG) kappa chain (Kotula and Curtis, 1991), human  $\beta$ -1,4-galactosyltransferase (Herrman et al, 1995), human gastric lipase (HGL) (Crabbe et al, 1996) and bovine pancreatic trypsin inhibitor (BPTI) (Parekh and Wittrup, 1997).

#### 1.5.1. Transformation in Yeast

It is obvious that any attempt to express heterologous genes from constructs in *S. cerevisiae* requires the initial step of introducing the foreign DNA into the cell.

Chemical methods can be utilized, for example lithium acetate can be used in the presence of polyethylene glycol (PEG) to transform intact cells (Ito et al, 1983; Gietz and Woods, 1994) or protoplasts can be made and transformed using calcium chloride and PEG (Hinnen et al, 1978). The latter method is more efficient by an order of magnitude but requires the embedding of protoplasts in selective agar

whereas intact transformants can be grown using straightforward spread plate procedures. More recently, high efficiency transformation of yeast has been achieved by electroporation (Becker and Guarente, 1991) which by means of an electric pulse of short duration renders the cell 'leaky' to macromolecules such as DNA. The advantages of electroporation are that it is simple, efficient, and that following transformation the membrane recovers and cells can be plated on selective agar in the usual way.

### 1.5.2. Yeast Vectors

There are many yeast vectors that can be used for cloning or when incorporating a suitable promoter (1.5.4), for gene expression. Such plasmids are often termed 'shuttle vectors' since they can be conveniently propagated and manipulated in *E. coli* before transformation into yeast for expression. The *E. coli* plasmid component usually consists of the origin of replication and a selectable antibiotic resistance marker from pBR322 for example or more usually nowadays from the pUC series of plasmids. pUC plasmids replicate at higher copy number than pBR322 giving higher yields in plasmid preparations from *E. coli* during subcloning procedures. Shuttle plasmids fall into four main types: yeast integrating plasmids (YIps), yeast centromeric plasmids (YCps), yeast replicating plasmids (YRps), and yeast episomal plasmids (YEps).

#### 1.5.2.a. Yeast Episomal Plasmids.

YEp vectors based on the yeast plasmid 2 $\mu$ m circle have the advantage of being stably propagated at high copy number within the cell. Generally, expression of a heterologous gene from a YEp results in a significant increase in protein production that is a consequence of increased gene dosage (Rose and Broach, 1990).



The 2 $\mu$ m circle is a small double stranded autonomously replicating plasmid naturally occurring within *Saccharomyces* at a copy number of between 60 - 100 (Broach, 1981). The plasmid contains two unique regions separated by two further sequences of 599bp that are exact inverted repeats of each other. The protein product of the coding region *FLP* plays a role in catalyzing recombination at specific sites lying within the inverted repeats. Recombination results in the inversion of the two unique sequences with respect to each other. This event is one arm of the plasmid's method of persistence since it enables amplification of copy number (and therefore compensates for loss of plasmids due to errors in mitotic segregation) by shifting the plasmid into a rolling circle mechanism of replication, thereby allowing several progeny to be produced from a single plasmid parent. Alternatively replication can proceed from the single origin (*ORI*) in both directions through an intermediate to produce two plasmid copies from a single parent. The second arm of the plasmid's mode of persistence is efficient equipartitioning during cell division. Two distinct regions *REP1* and *REP2* code for proteins crucial to this process. A third region (a sequence of tandem repeats of a 62bp element located near to the origin of replication) *REP3* or *STB* appears to function as a centromeric type element during host cell division (Rose and Broach, 1990).

2 $\mu$ m based yeast expression vectors all contain at minimum the *ORI-REP3* region which in these vectors functions as the *ARS* (autonomous replicating sequence), a segment of *E. coli* plasmid as previously mentioned, and a yeast selectable auxotrophic marker such as *LEU2*, *TRP1*, or *URA3* (see below). The products of *REP1* and *REP2* genes required for partitioning are supplied in a suitable *S. cerevisiae* host by endogenous 2 $\mu$ m circles. Generally a 2.2Kb *EcoRI* fragment or 2.1 Kb *HindIII* fragment from the B form of the 2 $\mu$ m circle encompassing one of the inverted repeats in



addition to the *ORI-REP3* region is utilised. A well known example of this type of vector is YEp13 which was constructed by cloning the 2.2Kb *EcoRI* 2 $\mu$ m fragment and a 4.0Kb *PstI* *LEU2* fragment into pBR322 (Broach et al, 1979). Two of the vectors employed in the current study (plasmids pG3 and p2UG) (Schena and Yamamoto, 1988; Picard et al, 1990) are also examples of this type of vector (2.4).

Early YEp vectors such as pJDB248 utilized the entire 2 $\mu$ m genome (Beggs, 1978). The advantage of such vectors is that they exhibit a greater degree of stability over a longer period, particularly in the absence of selective growth. However, the intact 2 $\mu$ m circle has an extremely limited number of non-disruptive insertional sites which can make plasmid construction problematic.

YEp continue to be the 'workhorses' for heterologous expression in yeast. Recent modifications include the design of vectors encoding glutathione-S-transferase (GST) affinity 'tags' which enable a fusion protein to be produced that is easily purified using an affinity column and glutathione-Sepharose. The tag is then easily removed from the protein by virtue of a protease cleavage site following purification (Ward et al, 1994).

#### 1.5.2.b. Yeast Centromeric Plasmids

YCps display high mitotic stability due to the presence of a yeast centromeric sequence (*CEN*) (Clarke and Carbon, 1980) that confers regular mitotic and meiotic segregation. The centromeric sequence also ensures low copy number and these plasmids are typically present at 1-2 copies per cell. This may be a disadvantage if overexpression of the heterologous gene product is required, but may be circumvented in part by the use of a powerful promoter. For purposes such as *in vivo* mutagenesis however, the low copy number may be an advantage. As little as 125bp of

yeast centromeric DNA is sufficient for efficient segregation (Stearns et al, 1990) but in practice YCp vectors tend to contain larger centromeric fragments. The centromere of chromosome IV (*CEN4*) is frequently used in YCp vectors.

It should be noted that circular plasmids containing centromeric sequences are not as stable as the host linear chromosomes and YCp vectors tend to be lost approximately once in 100 divisions compared to one in 10,000 divisions for natural linear chromosomes (Hartwell et al, 1982; Stinchcomb et al, 1982). This lower stability accounts for the presence in some cells of 2 rather than 1 copy of the plasmid. A higher copy number of YCps has been shown to be debilitating in yeast cultures and such events are selected against (Futcher and Carbon, 1986). YCps are autonomously replicating and contain an autonomously replicating sequence (ARS) that is usually chromosomally derived but may in some vectors be conferred by 2 $\mu$ m sequences. *ARS1* which is located next to the *TRP1* gene in yeast can be excised on an *EcoRI* fragment and is commonly utilized in YCps. *ARS2* is also often used but other ARSs are less convenient to manipulate (Hadfield, 1994). In common with YEps, YCps contain a selectable yeast marker and pBR322 or pUC plasmid sequences for shuttling purposes. YCp50 and its derivatives are examples of frequently used vectors of the centromeric type. YCp50 contains *CEN4* and *ARS1*, *URA3* as the selectable marker, and ampicillin and tetracyclin resistance markers from pBR322 (Stearns et al, 1990). pBM150 a vector employed in the current study is a YCp vector containing *ARS1* and *CEN1* in addition to the *URA3* gene and pUC18 sequences (2.4).

YCp vectors form the basis for the construction of yeast artificial chromosomes (YACs) which have become indispensable in physical gene mapping due to their ability to contain very large fragments of DNA (up to  $\approx$ 1Mb). Like



YCps YACs contain CEN and ARS sequences in addition to yeast selectable markers and sequences for selection and propagation in *E. coli*. Unlike conventional YCps however, they also contain a telomere at either end. DNA fragments are cloned between the two telomeric arms by restriction digest and subsequent ligation. The YAC is then transformed into yeast where it behaves like a 'mini chromosome' (Burke et al, 1987).

#### 1.5.2.c. Yeast Integrative Vectors

YIps, as the name suggests do not contain a yeast origin of replication; replication occurs by integration into sites within the yeast cell genome. Integration takes place by homologous recombination, whereby the integrative fragment replaces the homologous region in the host chromosome usually as a single copy and is then maintained with high stability (integrated sequences tend to be lost at a frequency of between 1/1000 and 1/10000 divisions (Stearns et al, 1990)).

When the YIp vector contains more than one yeast gene, for example when the gene to be expressed is a yeast gene manipulated *in vitro* together with the yeast selectable marker on the plasmid, then cleavage within one of the regions of homology has been shown to greatly enhance the frequency of integration in the vicinity of the cleavage site. Thus integration can be directed to the chromosomal locus of the manipulated gene or of the selectable marker as desired. When the vector contains a heterologous gene then integration can be accomplished by homology with the selectable marker.

The integration event following vector cleavage within one of the regions of homology results in duplication of the homologous sequences and therefore if the cloned gene is a yeast gene the copy number in the genome is effectively



doubled. This is obviously not the case with an integrated heterologous gene which has a copy number of 1.

Recombination events occurring after integration can result in complete excision of the YIp vector returning the host chromosome to the wild type state. This excision event can be extremely useful, since recombination *in vivo* can take place at any point within the stretch of homologous sequence shared by the duplicated segments. A mutation introduced *in vitro* into a cloned fragment may therefore, at low frequency, replace the wild type sequence but otherwise the host chromosome is returned by this post-integration recombination event to the wild type state (transplacement). Using a suitable selective marker such as *URA3* which can be both positively and negatively selected for allows both integration and excision events to be monitored. Colonies that have undergone the excision event can be screened for a mutant phenotype thereby greatly facilitating mutagenesis studies (Stearns et al, 1990).

The integrative plasmid pPIC9 (Invitrogen) used as an alternative in the current study and other similar vectors designed for use with *Pichia pastoris* are targeted to the methanol dependent alcohol oxidase 1 (*AOX1*) locus by homology with 5' and 3' flanking regions of the gene. The gene to be expressed is cloned between these two regions of homology and site specific replacement of *AOX1* with the heterologous gene can occur by a double recombination event (see 1.5.7).

Although expression levels achieved with the single gene dose typically associated with YIp vectors are usually sufficient it may be desirable to increase copy number. This has been achieved by targeting a specially designed YIp to the ribosomal DNA repeats which are present in the yeast genome at 140 copies. Plasmid pMIRY2 (multiple

integration into the ribosomal DNA in yeast) directed to the ribosomal locus by Lopes et al (1989, 1991) resulted in the successful integration of more than 100 copies of the YIp vector. Optimal production of a phosphoglycerate kinase using this vector reached as much as 50% total soluble intracellular protein. However, insertion of the heterologous gene was found to significantly reduce mitotic stability in comparison to the original vector. Overall plasmid size has been found to be an important determinant of mitotic stability in these vectors as well as the nature of the rDNA fragment contained within the vector for targeting integration (Lopes et al, 1996).

The multicopy (20-25) Ty1 transposable element present in the genome of most laboratory strains of *S. cerevisiae* has also been used as an integrative vector for heterologous expression. For example, human nerve growth factor has been expressed at multiple copy number in *S. cerevisiae* using the Ty1 element as a target site for integration (Sakai et al, 1991). The Ty1 element is also extremely useful for the production of fusion proteins that still assemble into Ty-virus like particles (Ty-VLPs) which are easily purified and also immunogenic. For these reasons, fusion Ty-VLPs are used both as a means of purifying heterologous viral proteins and as potential carriers for vaccines. Fusion products containing protein components of HIV such as *env*, *pol*, and *gag*, influenza virus haemagglutinin, feline leukaemia virus *env*, and bovine papillomavirus E1 and E2 have been produced (Kingsman et al, 1994).

#### 1.5.2.d. Yeast Replicative Plasmids

YRps are rarely used these days due to their extreme mitotic instability. Even under selective conditions the loss of these plasmids is of the order of 10% per generation (Hadfield, 1994). Autonomous replication in



these vectors is conferred by a chromosomally derived ARS such as *ARS1* and it is to be noted that the problem with these vectors is not due to inefficient replication (copy number may be high) but due to extremely poor segregation. A YRp may, however, become stably inherited due to an integration event via homology with the auxotrophic marker. Stability may also be conferred by addition of a centromere thereby effectively converting the YRp into a YCp.

### 1.5.3. Yeast Selective Markers

Crucial to any cloning exercise utilizing yeast vectors is the presence of a selectable marker to enable isolation of transformed colonies. The most frequently used markers are auxotrophic markers such as *TRP1*, *LEU2*, *HIS3*, *LYS2*, and *URA3* used in conjunction with the appropriate chromosomal mutation carried by an auxotrophic strain of *S. cerevisiae*. For example the *LEU2* gene, which can be manipulated as a 2218bp *SalI/XhoI* fragment (Hadfield, 1994), codes for the enzyme  $\beta$ -isopropylmalate dehydrogenase which complements the defective leucine gene of a *leu2-* auxotroph. The commonly used double frameshift mutation *leu2-3,112* is an example of a completely recessive and almost completely non-reverting disruption of the leucine gene (Hinnen et al, 1978).

*URA3* and *LYS2* have the double advantage of both positive and negative selection since positive selection is conferred by complementation of the suitable auxotrophic strain and negative selection conferred by growth in a suitable medium containing an inhibitor of cells expressing the wild type gene product. This can be particularly useful in situations such as the one previously mentioned where rare recombinational excision events resulting in the loss of the selectable marker can be screened for by negative selection. For *URA3* and *LYS2* this can be accomplished by growing cells on medium containing 5-



fluoroorotic acid or  $\alpha$ -aminoadipate respectively (Stearns et al, 1990).

Although auxotrophic markers are the most widely used selectable markers, dominant markers such as resistance to chloramphenicol or kanamycin can also be used. Such an antibiotic resistance marker can be used to select transformants in both yeast and *E. coli*. Inhibitory concentrations of antibiotic far higher than for selection in *E. coli* are required, for example  $\approx 2\text{mg/ml}$  of chloramphenicol as compared to  $\approx 10\mu\text{g/ml}$  for *E. coli* and furthermore these usually need to be determined empirically for yeast as strains differ in sensitivity (Hadfield et al, 1986; Hadfield et al, 1990).

#### 1.5.4. Yeast Promoters

In common with other eukaryotic promoters yeast promoters usually contain a TATA box (Goldberg-Hogness box) but this tends to be sited somewhat further away from the site of initiation of transcription than in other eukaryotes. The TATA box in a typical yeast promoter tends to be situated between 40 and 100 nt upstream of the transcription site as opposed to around 30 nt. This TATA box may not be essential for efficient expression of a gene but has a role to play in determining the initiation of transcription (Ogden et al, 1986). Cis-acting elements which are essential for expression and transcriptional control in yeast are, however, found in all yeast promoters. These sequences known as 'upstream activating sequences' (UASs) may be present as a single motif, multiple copies of the same motif, or as more than one component binding different trans-acting proteins each with a different role related to transcriptional control. Upstream activators also contain an 'activator core' sequence (AC) which has homology between different yeast promoters. UASs in yeast are

usually situated a considerable distance (at around position -500 of the 5' UTR for example) from the site of transcription initiation. Different yeast promoters may also contain additional upstream protein binding elements that have a more minor role in controlling expression. Repressor sequences may also be present. Figure 1.4 illustrates the structure of a 'typical' yeast promoter.

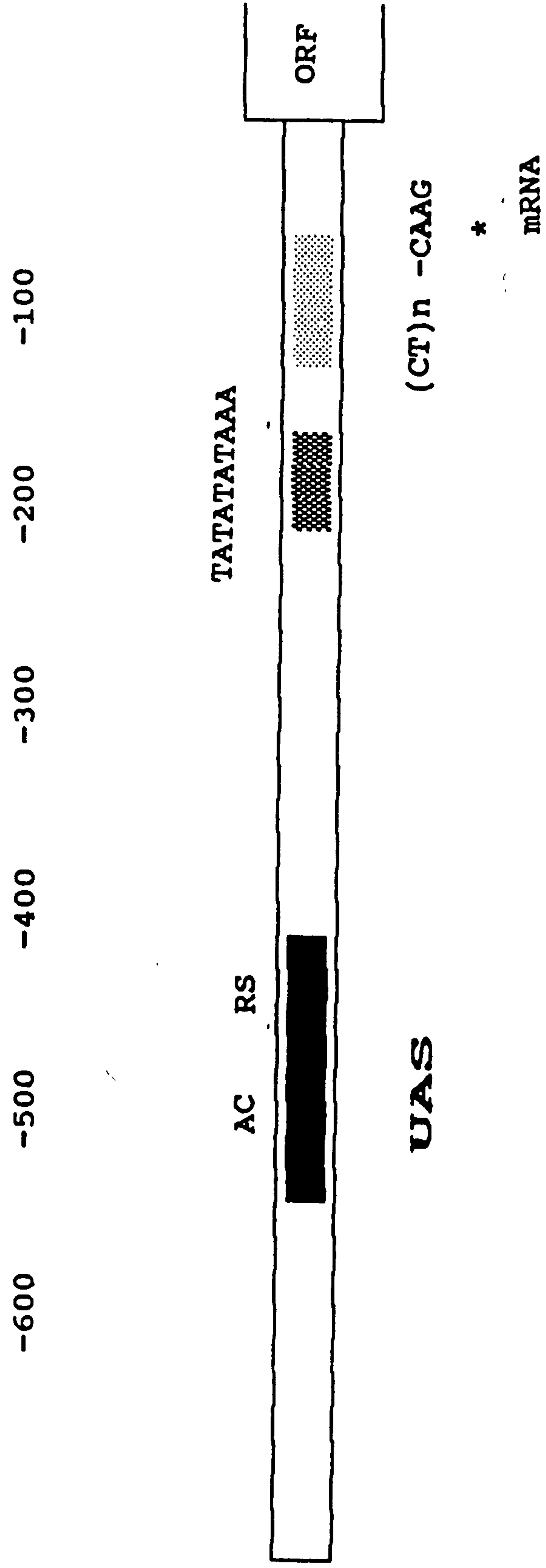
#### 1.5.4.a. Constitutive Promoters

Proteins involved in the glycolytic pathway such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH or GPD) or phosphoglycerate kinase (PGK) are among the most abundant proteins in yeast and individually account for approximately 5% of soluble proteins and mRNA in glycolytically growing cells. The promoters of such highly expressed genes make ideal candidates to drive high level constitutive expression of heterologous genes from yeast expression constructs. Indeed, both *GPD* and *PGK* promoters have been used widely to express a variety of eukaryotic proteins which include: hepatitis B surface and core antigens (Bitter and Egan, 1984; Kniskern et al, 1986), human superoxide dismutase (Hallewell et al, 1987), epidermal growth factor (Urdea et al, 1983), wild type and mutant  $\alpha_1$ -antitrypsins (Travis et al, 1985), goat  $\alpha$ -lactalbumin (Takeda et al, 1990), and human interferons (Tuite et al, 1982; Derynck, 1983), calf chymosin (Mellor et al, 1983), immunoglobulins (Wood et al, 1985), wheat  $\alpha$ -amylase (Rothstein et al, 1984) and HIV antigens (Adams et al, 1987) for *GPD* and *PGK* promoters respectively.

In the case of human superoxide dismutase and hepatitis B core antigen driven by the *GPD* promoter very high levels of expression have been achieved ( $\approx$  30% of soluble yeast protein) which compares favourably with the highest heterologous production levels observed in *E. coli*. It is an example of the empirical nature of heterologous

Figure 1.4. Structure of a 'typical' yeast promoter showing an upstream activating sequence (UAS) and the TATA box (after Kingsman et al, 1990). AC = activator core sequence; RS = repetitive sequence; ORF = open reading frame of downstream gene. A putative mRNA initiation point is also illustrated.





expression systems, however, that foreign protein production driven by the *GPD* promoter can vary by as much as 0.1 - 30% of the soluble cell protein depending on the protein expressed (Rosenberg et al, 1990). It has also been noted that the levels of heterologous expression achieved with the *PGK* promoter where levels are typically 1 - 5% of total cell protein using the promoter on a high copy number YEp that yields of heterologous product are lower than the yield of homologous *PGK* protein obtained using the same promoter and construct (Mellor et al, 1985). It has been suggested that transcription of heterologous genes may be less efficient due to the absence of certain enhancer sequences in *PGK* (Mellor et al, 1987). Another plausible explanation is that *PGK* may be turned over in the cell more slowly than many foreign gene products.

Both promoters *GPD* and *PGK* contain key sequences including essential UAS regions. It has been shown that a minimum length of flanking 5' sequence is required for efficient expression since the UAS region of both promoters resides  $\approx$  500bp upstream from the site of transcriptional initiation. A shorter 400bp segment has been shown in the case of the *GPD* promoter to result in a drastic reduction of expression (50 - 100 fold) compared to constructs containing longer segments of promoter (Rosenberg et al, 1990). Bitter and Egan (1984) suggest that  $\approx$  675bp of *GPD* 5' UTR is required for efficient transcription to occur. There are three *GPD* genes in *S. cerevisiae* (Rosenberg et al, 1990) but the most abundant *GPD* protein is expressed from *GAP491* (McAlister and Holland, 1983) and it is the *GAP491* 5' UTR that is generally referred to in terms of heterologous gene expression. Plasmid pG3 (Figure 2.1), a constitutive YEp vector used in the current study, carries a 650bp *TaqI* fragment of the *GAP491* 5' UTR (Musti et al, 1983; Schena et al, 1991).

Both *GPD* and *PGK* promoters contain a TATA box between the

transcription start site and UAS regions. A pyrimidine rich tract in the *PGK* promoter surrounding the mRNA start site is thought to mediate the efficiency of initiation in this particular promoter.

The UAS of the *PGK* promoter has been shown to contain three repeats of the sequence CTTCC in addition to the AC motif and also contains another protein binding site 5' to the UAS region that seems to have a role in controlling expression in response to carbon source although this regulation is not tight, a difference in expression levels between acetate or pyruvate sources compared to glucose differing only by  $\approx 30$  fold (Kingsman *et al*, 1990). Thus although the *PGK* promoter is generally thought of as being constitutive it does contain an inducible-like element. This protein binding site has been shown to be a modulator of rather than essential for activation of transcription (Stanway *et al*, 1987). Interestingly, the *PGK* promoter also contains a heat shock response element which has been demonstrated to be responsible for a small transient increase in expression following heat shock (Piper *et al*, 1988).

The alcohol dehydrogenase I (*ADHI*) promoter, another constitutive metabolic promoter that has been successfully employed to drive the expression of heterologous genes is similar to the *PGK* promoter in that expression levels can differ by  $\approx 10$  fold depending on the carbon source (Denis *et al*, 1983). The *ADH1* promoter thus contains an inducible-like element. Inducible promoters proper however, exhibit a far more drastic response in terms of gene expression to specific environmental stimuli and can be used for extremely tight regulation (see 1.5.4.b). The *ADH1* promoter has been used successfully for the expression of human IFN- $\alpha$ , for example, (Hitzeman *et al*, 1981) and more recently for the expression of rat fatty acid synthase (Kupfer *et al*, 1996).



The potential of various yeast gene promoters to drive high level heterologous expression is continually being examined. For example the powerful constitutive promoter of the yeast plasma membrane H<sup>+</sup> ATPase gene has been used express other heterologous ATPases in *S. cerevisiae* (Villalba et al, 1992; Mahanty et al, 1994) and has now been employed to express the cystic fibrosis transmembrane conductance regulator (CFTR) protein in yeast (Huang et al, 1996).

#### 1.5.4.b. Inducible Promoters

It may be that the foreign gene product has a debilitating effect on yeast cell growth and if this is the case then a regulatable rather than a constitutive promoter may be used to achieve high biomass before expression of the gene product. If the heterologous product is highly toxic to the cell then, of course, a regulatable promoter is not simply desirable but essential.

Perhaps the best known examples of regulatable promoters are those attached to the genes encoding the enzymes of galactose metabolism. In the absence of glucose, *S. cerevisiae* can utilize galactose as a precursor of glucose for use in the glycolytic pathway. The conversion of galactose depends upon three enzymes: galactokinase, galactotransferase, and galactose epimerase. The genes encoding these enzymes (*GAL1*, *GAL7*, and *GAL10* respectively) are regulated by the products of two further genes, *GAL4* and *GAL80*, the former acting as a positive transcriptional activator of the three enzyme genes and the latter as a repressor by inhibition of the *GAL4* protein (Schneider and Guarente, 1991). The *GAL4* protein, which is present in wild type cells at very low concentrations, contains a single zinc finger binding motif that binds to UAS regions in the promoters of *GAL1*, *GAL7*, and *GAL10* that contain a

common region of dyad symmetry suggesting that *GAL4* binds as a dimer (Verdier, 1990). Once bound, transcriptional activation is thought to be brought about through interaction between a highly negatively charged transcriptional activating domain of the *GAL4* protein and the C-terminal domain of the large subunit of RNA polymerase II, in a similar fashion to the postulated mode of action of other protein molecules acting as transcription factors. In the absence of galactose the *GAL80* protein binds *GAL4* protein thereby preventing transcription of the *GAL* enzyme genes. In the presence of galactose a derived metabolite causes dissociation of *GAL4*-*GAL80* to occur, effectively freeing *GAL4* to act as a transcription factor. *GAL4* also binds to the UAS of *GAL80* promoting transcription of its own regulator and so there is a continuing requirement for galactose in the medium to induce expression of *GAL1*, *GAL7*, and *GAL10* (Verdier, 1990). Recent evidence suggests that the initiation of *GAL80* transcription involves two separate pathways one of which is constitutive and *GAL4* and TATA independent, requiring only the presence of a 16 bp initiation region surrounding the +1 site and the other of which is *GAL4* regulated and dependent on the TATA box (Sakurai et al, 1994).

This elegant regulation of sugar metabolism can be employed to regulate the expression of heterologous genes: induction by galactose in the growth medium, repression by glucose (Guarente et al, 1982; Johnston and Davies, 1984). In fact the *GAL1*, *GAL7*, and *GAL10* promoters all exhibit a rapid and drastic response to a specific switch in carbon source. In the absence of galactose transcriptional activity is virtually zero but the addition of galactose to a medium without glucose results in very high levels of transcription. The expression of the *GAL1* or *GAL10* genes has been estimated to be induced by  $\approx 2000$  fold in the absence of glucose and presence of galactose (Kingsman et al, 1990). The *GAL* promoters have been utilized for the



controlled expression of many heterologous proteins including HSA (Kingsman et al, 1990), calf chymosin (Goff et al, 1984) and more recently a human phosphomannose isomerase (Proudfoot et al, 1994), human fibrinogen (Roy et al, 1995) and rat apolipoprotein E (Nomura et al, 1995).

Plasmid pBM150 (Johnstone and Davies, 1984) (Figure 2.1), a centromeric vector utilized in the current study contains promoter sequences from the *GAL1* and *GAL10* genes. Since these two promoters share a common UAS (Verdier, 1990) and transcribe in opposite orientations, vectors such as pBM150 containing the complete *GAL1-GAL10* promoter fragment can be used to simultaneously express two heterologous gene products. Carlson (1988) used pBM150 to simultaneously express both heavy and light chain immunoglobulins in yeast. A single product can be expressed from either promoter depending on the restriction site chosen for subcloning (2.4).

Unlike the promoter of the *ADH1* gene which is to all intents and purposes constitutive the promoter of the alcohol dehydrogenase 2 gene (*ADH2*) is tightly regulated by glucose repression. The *ADH2* promoter contains two UAS regions which are responsible for the inducible nature of this promoter, although it should be noted that either of the UAS elements alone are capable of conferring regulation by glucose on a heterologous promoter. However, both UAS elements act synergistically to maximum effect. A transactivator (ADR1), acting in a similar fashion to the *GAL4* protein, and containing two zinc finger binding motifs, has been shown to bind specifically to one of the UAS elements in the *ADH2* promoter with consequent enhancement of transcription (Denis et al, 1981). Unlike the *GAL* inducible promoter system, however, which requires not only the absence of glucose but the presence of galactose for transcriptional activation, the *ADH2* promoter can be activated simply by the depletion of glucose from



the medium. Typically cultures are grown for  $\approx$  24hr well into stationary phase such that glucose becomes depleted in the medium  $\approx$  10hr prior to harvesting (Price et al, 1990).

It has been suggested by Denis and Gallo (1986) that the ADR1 transactivator may be a phosphoprotein, the phosphorylation or dephosphorylation of which and accompanying activation status, may be mediated by glucose repression. In fact in the presence of glucose transcription levels of ADH2 are undetectable, regulation is so tight, but depletion of glucose results in significant derepression, such that ADH2 protein levels quickly rise to  $\approx$  1% of soluble cell protein (Price et al, 1990). Glucose repression of the ADH2 promoter is thought to be due directly to a lack of activation by ADR1 rather than to the action of a repressor protein.

The ADH2 promoter is thus another ideal inducible promoter that can be used for regulated heterologous expression in yeast with the additional advantage that depletion of glucose, which occurs naturally throughout the growth curve, is the mechanism of induction thereby obviating the need to switch growth media, or to add inducing compounds, or alter the culture temperature as is required of other inducible promoters. The ADH2 promoter carried on YEp vectors has been used for the successful expression of several cytokines, for example both bovine and human interleukin 2 (Price et al, 1990).

The promoter from the yeast copper metallothionein gene *CUP1* (Fogel and Welch, 1982; Karin et al, 1984) also contains two cis-acting UAS elements (UAS<sub>p</sub> and UAS<sub>d</sub>) (Thiele and Hamer, 1986) both containing an inverted repeat of the sequence TCTTTTGCT and is induced by addition of copper ions to the medium. Both elements are required for maximal gene expression. The gene product copper metallothionein counteracts the toxic effect of copper by acting as a

chelator and it has been reported that yeast responds to the selective pressure of copper by amplification of *CUP1* (Etcheverry, 1990). Despite the fact that the UAS regions which bind transactivating proteins have been defined, other sequences still further upstream appear to be essential for induction by copper since shorter *CUP1* promoter segments retaining both UASs have been shown to result in constitutive expression (Etcheverry, 1990).

Successful inducible expression using the *CUP1* promoter depends upon determining the optimal concentration of copper for induction since natural strain tolerance of copper varies. Too high a concentration will cause inhibition of translation due to toxic effects; too low a level of copper will not act as a potent inducer because the copper is scavenged by endogenous metallothionein.

The *CUP1* promoter has been used for the successful expression of human tissue plasminogen activator (Pennica et al, 1983) as well as HSA (Etcheverry, 1990), rabbit liver cytochrome P-450 3a (Fujita et al, 1990), mouse Ig kappa chain (Kotula and Curtis, 1991), and the leech anticoagulant hirudin (Hottiger et al, 1994).

The acid phosphatase gene family of yeast consisting of at least three genes (*PHO3*, *PHO5*, *PHO11*) display differential expression in response to the level of inorganic phosphate in the medium. The *PHO5* and *PHO11* genes are both induced by low concentrations of phosphate and the promoter of the former has been successfully utilized in the inducible expression of exogenous genes, for example hepatitis B surface antigen (Mijanohara et al, 1983). Similar to the elegant regulatory system of the *GAL* gene family, at least two transactivators *PHO4* and *PHO2* are known to play a role in the transcriptional activation of *PHO5*, the promoter of which, contains dyad symmetric sequences with homology to the yeast UAS consensus core (Rudolph and Hinnen, 1987).



The *PHO5* promoter is also subject to negative regulation by two further proteins *PHO80* and *PHO85* which are thought to bind to the transactivator *PHO4* in the uninduced state (Rudolph and Hinnen, 1987).

Very recently an inducible yeast expression vector was constructed using the promoter of the *S. cerevisiae SOR1* gene which encodes sorbitol dehydrogenase. Expression of heterologous proteins is induced when cells are grown in the presence of sorbitol (McGonigal et al, 1998).

It is important to note that a constitutive promoter can be made regulatable by the insertion of the critical UAS elements into a normally constitutive promoter. For example, the shared UAS from the *GAL1-GAL10* gene was engineered by Kingsman et al (1990) into a deletion derivative of the *PGK* promoter which lacked its native UAS making it inducible by galactose. Deleted derivatives of the *GPD* promoter which are demonstrably inactive due to the absence of the critical UAS region can be made functional and inducible by the insertion of UAS regions derived from other promoters such as *GAL1-GAL10* or *ADH2* (Cousens et al, 1987; Rosenberg et al, 1990).

Efficient control of a yeast promoter can also be achieved through hormonal regulation. It is known that mammalian steroid receptors remain active as conditional regulators of transcription when expressed in *S. cerevisiae* (Metzger et al, 1988; Schena and Yamamoto, 1988) and construction of a responsive promoter can be achieved by fusion of three tandem 26 base pair glucocorticoid response elements (GREs) upstream of the promoter. Transformation of a second vector encoding the glucocorticoid receptor into the yeast cell then provides all the necessary elements for steroid control of heterologous gene expression since addition of glucocorticoids results in specific binding of receptor to the response elements in the promoter with consequent



transcriptional enhancement. Plasmid p2UG utilized in the current study (Figure 2.2) is an example of a steroid inducible vector containing the three GREs fused upstream of the yeast cytochrome c gene(CYC1) promoter region. Picard et al (1990) have reported up to a 100 fold increase in expression of the bacterial chloramphenicol acetyltransferase gene from p2UG with the addition of deoxycorticosterone to 10 $\mu$ M in the culture medium compared to uninduced cultures.

#### 1.5.5. Factors Affecting Optimal Expression in Yeast

##### 1.5.5.a. Gene Dosage

It has been mentioned previously that in general the level of expression of a foreign protein in a yeast is a function of gene dosage: the greater the number of copies of a gene, the higher the level of expression. For this reason, high copy number vectors such as YEps containing the 2 $\mu$ m origin of replication typically yield an increase in production over single copy vectors that is commensurate with their increased copy number. For example, it has been demonstrated that expression levels (measured by specific activity) of  $\beta$ -isopropylmalate dehydrogenase and orotidine 5'phosphate decarboxylase (the protein products of *LEU2* and *URA3* respectively) are approximately 30 fold higher when expressed on a YEp rather than a YCp (Rose and Broach, 1990). Similar increases in production are seen using multiple integration techniques such as YIp targeting to the ribosomal locus (Lopes et al 1991).

Assuming that expression is not limited by other factors (see below) techniques are available for increasing gene dosage and concomitant expression levels still further using YEp vectors. Standard YEp vectors have a copy level in yeast strains containing endogenous 2 $\mu$ m circles (*cir<sup>+</sup>* strains) of  $\approx$  50 copies, although it may be higher or lower

(10-100) (Rose and Broach, 1991; Kingsman et al, 1991). This copy number can be drastically increased by use of a fragment of the *LEU2* gene which lacks its promoter. Expression from this truncated gene (*leu2-d*) is minimal compared to expression levels from *LEU2*. YEps containing the *leu2-d* allele are maintained at a copy number between 200-300 since plasmid amplification is driven by the requirement of the auxotrophic host cell to achieve higher levels of  $\beta$ -isopropylmalate dehydrogenase.

An undesirable level of recombination can occur between transforming vectors which carry the entire 2 $\mu$ m circle genome and endogenous 2 $\mu$ m circles which can often result in the loss of the heterologous gene. For this reason strains lacking endogenous circles (*cir*<sup>-</sup> strains) are preferred and furthermore because the entire capacity of the host cell is available for the maintenance of the transforming vector. The use of the promoterless *leu2-d* gene and *cir*<sup>-</sup> strains can result in the amplification of a YEp vector to extremely high levels of 400-500 copies/cell (Johnston et al, 1986). Using such a system in conjunction with the *ADH2* promoter Price et al (1990) reported levels of ADH2 protein that reached  $\approx$  20% of total cell protein. It should be noted that *cir*<sup>-</sup> strains cannot be used for vectors that lack the *REP1* and *REP2* genes (1.5.2.a).

Expression levels of FLP, the site specific recombinase, that promotes amplification of endogenous 2 $\mu$ m circles can also be increased by use of *FLP* containing YEp constructs. Additional FLP activity can result in plasmid amplification to 200-400 copies/cell (Rose and Broach, 1990).

Such techniques which significantly raise heterologous gene dosage levels can be used to optimise production or enhance expression in situations where levels are poor. However, many factors influence final levels of expression and gene amplification may not necessarily result in higher



levels of production. Furthermore, a foreign gene which is deleterious to host cell growth will still require the use of an inducible promoter to achieve significant levels of expression.

#### 1.5.5.b. Efficiency of Transcription

In order for efficient transcription to occur the heterologous gene should be fused to a suitably powerful yeast promoter which contains all the necessary elements for transcription initiation. Constitutive and inducible promoters that have been successfully employed for heterologous gene expression in *S. cerevisiae* have already been discussed (1.5.4). Often levels of expression achievable using a suitable promoter are sufficient; however, it is worth noting that transcription can be enhanced still further by manipulation of promoter sequences to create artificial promoters that are more powerful than the already potent promoters from which they are derived. An example is the *GRAP1* promoter constructed from elements of the *GAL7* and *ADH2* promoters. This artificial promoter contains the two UAS regions from the *GAL7* promoter combined with the downstream initiation region from the *ADH2* promoter (Leplatois et al, 1992).

The *CYP1* protein is a transcriptional regulator which is known to bind to UAS sequences in the *CYC1* promoter and also binds to UAS regions in other cytochrome gene promoters. It contains a zinc finger DNA binding domain and has been shown to have preferential consensus sequences for binding. Perhaps significantly for potential improvements in the transcription of heterologous genes from the *CYC1* promoter, for example, it has recently been demonstrated that *CYP1* protein has a higher affinity for a UAS sequence CGGNNNTANCGG that is not found in its target promoter sequences *in vivo* (Nait-Kaoudjt et al, 1997). Greater understanding of transcriptional enhancement should



lead to the increasing design of artificial UAS sequences that are optimised for transcription above that of the wild type promoter.

Further enhancement of transcription may also be achieved by manipulation of limiting factors. A limiting factor may still prevent higher levels of expression following manipulations such as increase of gene dosage that should in theory lead to better production. For example the availability of the GAL4 protein within the cell is a limiting factor for GAL promoter constructs. Furthermore, this problem cannot be circumvented by simply increasing endogenous production of GAL4 because this leads to constitutive rather than inducible expression due to insufficient amounts of GAL80 available to bind GAL4 (Johnston et al, 1986). Schultz et al (1987) provided a solution to this problem by constructing a strain of *S. cerevisiae* carrying both the chromosomal GAL4 locus and an expression vector containing the GAL4 gene fused to the GAL10 promoter integrated into the genome at the HIS3 locus. Thus the increased amounts of GAL4 protein needed to drive still higher levels of transcription are subject to induction by galactose in the same manner as the heterologous gene carried on another GAL10 vector. This effectively removes GAL4 as a limiting factor when multiple copies of the GAL10 promoter are employed whilst still retaining the advantages of an inducible system. Following induction on galactose levels of GAL4 protein were shown by Schultz et al (1987) to increase 60 fold over wild type levels and expression of a heterologous protein (Epstein-Barr viral glycoprotein) to be enhanced by an order of magnitude.

In a similar fashion the ADR1 transcription factor may become a limiting factor when overexpression from the ADH2 promoter is desirable. As with GAL4 one means of increasing ADR1 levels is simply to express it from a

strong promoter but glucose repression of the *ADH2* promoter is, in part at least, lost (Irani et al, 1987). This problem can be circumvented by constructing a strain of *S. cerevisiae* containing an integrated copy of the *ADR1* gene fused to an inducible promoter such as the *ADH2* promoter. Excess *ADR1* which results in enhanced expression of proteins from the *ADH2* promoter thus becomes subject to control by the same inducible promoter (Price et al, 1990).

#### 1.5.5.c. Stability of Heterologous mRNA

The amount of heterologous transcript available for translation within the cell will obviously contribute significantly to the overall level of product produced. This amount clearly depends upon the efficiency with which the heterologous sequence is transcribed from the promoter (1.5.4 and 1.5.5.b) and also factors affecting the efficiency of translation (1.5.5.d) but will also depend upon the half life of the heterologous mRNA. Endogenous mRNA half-lives have been shown to vary between 1 min and 1 hr in *S. cerevisiae* (Losson et al, 1983; Santiago et al, 1986; Herrick et al, 1990). Clearly a heterologous mRNA that is very rapidly degraded may drastically reduce the final amount of protein product produced. However, the factors which determine transcript stability are poorly understood. It is known that the 3' polyadenine tail attached to the message following transcription is important in eukaryotic mRNA stability, together with the 5' methylated cap, and various structural elements either primary or secondary within the transcript itself that interact with mRNA binding proteins in regulating mRNA turnover (Ross, 1995). Yeast mRNAs are typically eukaryotic, consisting of a 5' cap and 3' poly A+ tails, although the number of adenine residues is generally lower than that seen in higher eukaryotes (McLaughlin et al, 1973). What is clear, however, is that heterologous signals for termination of transcription often fail to be



recognised by the *S. cerevisiae* transcriptional machinery, which results in the production of long and highly unstable transcripts (Zaret and Sherman 1984). For this reason most expression vectors for use with *S. cerevisiae*, in addition to a suitable promoter 5' to the polycloning site also contain transcription termination sequences derived from endogenous genes such as *PGK* or *ADH1* placed 3' to the cloning site. Vectors are often referred to as expression 'cassettes' with the gene for expression slotting in and out of a polycloning site placed between the yeast promoter and signals for transcription termination and mRNA polyadenylation.

#### 1.5.5.d. Efficiency of Translation

Subcloning of a cDNA fragment into a suitable vector under the control of a strong promoter will generally result in the overexpression of the desired mRNA which if sufficiently stable (1.5.5.c) will be available for translation. In certain cases poor levels of translation can inhibit good production from an overexpressed and stable mRNA (Smith et al, 1985; Scarpulla and Nye, 1986).

There are several factors which influence the efficiency of translation of foreign mRNAs in yeast which are presumably related to the natural preferred translational context. In higher eukaryotes the 'scanning model' of translation is the putative mechanism of translation initiation and it thought that a similar mechanism exists in yeast. The ribosome binds the 5' of the transcript and scans along its leader length until an AUG initiation codon is reached. However, many higher eukaryotic transcripts have leader sequences which contain a number of AUG codons upstream of the true initiation AUG. Translation in higher eukaryotes is tolerant of these 'false starts' and sequences in which the true initiation codon is embedded are recognised. Studies of AUG context in mammals suggest that the sequence

5'CACCAUGG3' is the optimal sequence for the initiation of translation (Kozak, 1984; 1986). This context differs in yeast in which a consensus start sequence 5'AA<sup>y</sup>AA<sup>a</sup>AAUGUCU3' is often seen. Alternatives to the mammalian consensus sequence were shown by Kozak (1986) to reduce translational efficiency of the preproinsulin gene by 20 fold. In contrast the consensus sequence appears to be far less important to the translational initiation process in yeast since alterations to it have a far less drastic effect. For example, only alteration of the favoured A nucleotide at the -3 position had any effect on the translational efficiency of *HIS4* and this only by a factor of two (Cigan *et al*, 1988).

The overriding mechanism in the initiation of translation in yeast is the first AUG codon scanned by the ribosome. This is perhaps not surprising since translation initiation in  $\approx$  95% of yeast transcripts occurs at the first AUG codon encountered (Cigan and Donahue, 1987). For this reason the presence of upstream AUG codons in foreign transcript leaders can severely reduce levels of translation in yeast and are best removed from cDNAs prior to subcloning. The introduction of AUG false starts to the leader region of *HIS4* and *CYC1* genes effectively disrupt translation, although some residual level of translation has been observed at the correct initiation codon (Sherman *et al*, 1980; Donahue and Cigan, 1988; Cigan *et al*, 1988). The 5' UTR of the *Ace* gene contains several AUG codons prior to the initiation codon proper (Hall and Spierer, 1986) and to avoid translational difficulties these were removed from the cDNA by exonuclease digestion from the 5' end (Hall, *pers comm.* 1991) before subcloning.

Secondary structure in the leader region of a transcript also has a significant effect on translational efficiency. Stem-loop structures in the 5' UTR can effectively block translation by preventing efficient binding of the ribosome



or by interrupting its migration (Kozak, 1986). Studies in yeast have shown that the insertion of sequences of dyad symmetry into *HIS4* and *CYC1* leader regions severely disrupt translation leading to a decrease in gene product to less than 5% of normal levels (Cigan et al, 1988; Baim and Sherman, 1988). Furthermore it has been shown that yeast appears to be more sensitive than mammalian cells to the occurrence of dyad symmetric or GC rich sequences (or of course both) in the leader sequence since the insertion of similar sequences into leader sequences of mammalian genes expressed in mammalian cells does not result in severe disruption of translation. Only when longer sequences of much higher GC content are inserted is translation significantly affected in mammalian cells (Pelletier and Sonenberg, 1985; Kozak, 1986).

Thus the presence of secondary structure may be of crucial importance with regard to heterologous expression, since higher eukaryotic genes may be significantly more GC rich in the 5' UTR in contrast to natural yeast leader regions in which the bias is towards A nucleotides and secondary structure tends not to be significant. Kupfer et al (1996) found that despite a high rate of transcription of recombinant rat fatty acid synthase (FAS) in *S. cerevisiae* translation was inhibited. Replacement of the 86 bp of 5' leader in the rat FAS cDNA with 5' UTR sequence from the *S. cerevisiae* ADH1 gene resulted in successful translation of the heterologous mRNA. Different levels of translational inhibition have also been shown to be influenced not only by the stability but also by the position of stem loops in the transcript leader, the formation of a stem loop closer to the start codon being more inhibitory than one near the 5' end of the leader (Oliveira et al, 1993).

Trimming of such 'foreign' leader sequences is therefore recommended for successful heterologous expression in yeast, especially since it is known that the length of the

leader sequence has little effect on translational efficiency. Baim and Sherman (1988) reported efficient translation initiation in *CYC1* with a leader sequence consisting of only 11 nt. The *ADH1* 5' UTR sequence used to improve translation of rat FAS mRNA in yeast was a nonamer (Kupfer et al, 1996). Generally, yeast leader lengths between 40 and 100 nt result in wild type levels of expression (Cigan et al, 1988). In common with higher eukaryotes leader length varies between 10 and 550 nt with an average length of 52 nt (Cigan and Donahue, 1987; Kozak, 1984) and supports the scanning model of translation.

#### 1.5.5.e. Stability of Protein Product

Assuming that all of the preceding requirements have been satisfied and that high levels of translated protein product are being produced, the yield can still be 'sabotaged' even at the final stage of cellular production, since any protein synthesised within the cytoplasm is liable to rapid degradation by vacuolar proteases when cells are harvested and lysed.

Known proteases that are found within the vacuole of *S. cerevisiae* include the two endoproteinases, protease A (PrA) and protease B (PrB), two carboxypeptidases, carboxypeptidase Y (CpY) and carboxypeptidase S (CpS), and two aminopeptidases, aminopeptidase I (API) and the  $\text{Co}^{2+}$  dependent aminopeptidase (Jones, 1984; Achstetter and Wolf, 1985). Protease inhibitors such as PMSF and EDTA will to some extent alleviate the problem but the construction of mutant strains containing disrupted protease genes has been very useful in the development of *S. cerevisiae* as an expression system. One of the most useful of these genetic 'disruptions' is the *pep4* mutation. *PEP4* encodes the precursor of PrA, which in turn is known to be involved in the activation of both PrB and CpY. *pep4-3* mutants contain a nonsense mutation (UGA) (Jones et al, 1989) in the



structural *PEP4* gene. PrA is effectively eliminated and the activity of both PrB and CpY severely inhibited. It has been demonstrated that PrB can still become reactivated in these mutants however (Moehle, 1988), and so often a double mutant containing a mutation in the PrB structural gene itself in addition to the *pep4-3* mutation is employed. The *S. cerevisiae* strain BJ2168 utilized in the current project carries the mutations *pep4-3* and *prb1-1122* and so is protease deficient. The *prb1-1122* allele is a nonsense mutation (UAA) (Zubenko et al, 1980). Use of such mutant strains has been shown to significantly improve the yield and quality of heterologous proteins (Cabezón et al, 1984; Rosenberg et al, 1984). Disruption of *YAP3* which codes for an endogenous aspartyl protease has been shown to reduce the degradation of HSA when it is expressed in *S. cerevisiae* (Kerry-Williams et al, 1998).

Perhaps a more significant problem in terms of difficulty is the actual half-life of the heterologous protein within the cell. There is no real solution to the possibility that a protein may be very rapidly degraded following translation. However, it is known that in eukaryotes, including *S. cerevisiae*, that a ubiquitin mediated proteolytic pathway has a major role in cytoplasmic protein turnover. Ubiquitin is a highly conserved protein of 76 amino acids which becomes covalently conjugated to a wide variety of intracellular proteins (Wilkinson et al, 1980) and appears to function as a mechanism for the recognition of damaged, denatured, or abnormal proteins which are then subsequently rapidly degraded by proteolysis (Hershko et al, 1982). Obviously a heterologous protein may be recognised as such by the ubiquitin system.

The conjugation, mediated by ubiquitin carrier proteins, is achieved by means of an amide bond between an activated carboxyl terminus of ubiquitin and amino groups on target proteins (Wilkinson, 1990). Specific ligases such as the

E3 ligase appear to mediate conjugation of ubiquitin to damaged proteins. Denatured, oxidised, and prematurely terminated proteins are known to be the preferred substrates of the ubiquitin dependent system. Damaged protein-ubiquitin conjugates are then degraded to free amino acids and ubiquitin by an ATP dependent protease with a specific binding site for ubiquitin. Experiments using yeast mutants defective in the breakdown of mutated and misfolded CpY protein have suggested that misfolded proteins in the ER may be core glycosylated before transport back to the cytoplasmic side of the ER where ubiquitin conjugation and subsequent degradation takes place (Hiller et al, 1996). Interestingly, many normal proteins are also bound by ubiquitin and the system seems also to function as a post-translational regulatory mechanism in normal protein turnover. The nature of the N-terminal amino acid is known to be an important determinant of degradation rate (Bachmair et al, 1986) i.e proteins may be stabilised or destabilised, in response to intracellular signalling, by addition of a suitable amino acid, such as Arg, to the N-terminus. Furthermore, once bound, ubiquitin-protein conjugates are not always directed through the protease degradation pathway, very often the conjugate is deconstructed to free ubiquitin and intact protein (Hershko et al, 1984). It has been postulated that the ubiquitin dependent system allows the cell to randomly 'proofread' protein structures before releasing an undamaged protein for functional purposes (Cox et al, 1986).

Clearly the fate of a heterologous protein may be significantly affected by ubiquitination. It may be particularly susceptible to degradation because it has been incorrectly processed by the yeast cell machinery and is denatured or prematurely terminated for example, and thus becomes a target for the action of the E3 ligase. It may simply become a target because it is a 'foreign' protein



and therefore recognised as abnormal by the cell. It may be misfolded in the lumen of the ER and transported back to the cytoplasm for degradation. The nature of the N-terminal amino acid of the protein is also clearly vital.

In *E. coli* the N-terminal methionine is always removed by methionine aminopeptidase (Ben-Bassat et al, 1987) with subsequent exposure of the penultimate amino acid. In *S. cerevisiae* this is not always the case. Cleavage of the N-terminal methionine appears to be dependent on the nature of the penultimate amino acid. If retained the N-terminal methionine may be acetylated and if cleaved the adjacent amino acid may also be acetylated (Sherman et al, 1986). Since the identity of the N-terminal residue can have a drastic effect on protein turnover by the ubiquitin dependent system substitution at the amino terminus may greatly increase protein stability in the cytoplasm. This can be achieved by expressing the protein under study with the desired N-terminus as a carboxyl terminal extension of ubiquitin. Following efficient processing by ubiquitin carboxyl hydrolases *in vivo* the recombinant protein is released with the engineered amino acid at the N-terminus. High level expression of human interferon (IFN) and  $\alpha$ PI (proteinase inhibitor) proteins with authentic N-termini have been reported using this technique (Sabin et al, 1989). In this way it has also been demonstrated that the half life of  $\beta$ -galactosidase can vary from 2 min (with Arg at the N-terminus) to over 20 hr (with Met, Ser, Ala, Thr, Val, or Gly) (Bachmair et al, 1986; Butt et al, 1988).

It may be worth considering engineering of the adjacent N-terminal amino acid of the protein to be expressed. There are two potential adjacent N-terminal residues for the DACH<sub>E</sub> protein since the signal peptide may or not be cleaved by *S. cerevisiae*. If the signal peptide of preacetylcholinesterase is not cleaved in *S. cerevisiae* to release the mature protein then the penultimate amino acid

would be an Ala residue. If the signal peptide is cleaved correctly then the N-terminal would be Val (Fournier et al, 1989). Both these residues are associated with increased stability. Furthermore, studies of cotranslational N-terminal processing would predict removal of the initiator methionine in growing polypeptide chains in which Ala or Val are present in the penultimate position. In the case of Ala acetylation of the N-terminus would also be predicted (Sherman et al, 1986; Huang et al, 1987). It is of course entirely possible that the presequence of DACE may be misprocessed by the signal peptidase in *S. cerevisiae* leading to an unpredictable N-terminus.

#### 1.5.6. Secretion of Heterologous Proteins in Yeast

The evidence suggests that the rapid proteolysis by means of the ubiquitin dependent system occurs in the cytoplasm. Therefore secretion of a heterologous protein may be desirable as a means of avoiding conjugation by ubiquitin by prior translocation into the secretory pathway. However, it should be noted that many non-secreted proteins even with a suitable secretion signal attached do not translocate rapidly enough to avoid such problems (Emr, 1990).

Secretion of heterologous proteins to the culture medium has many advantages including avoidance of degradation during extraction procedures by cellular proteases and simplified purification procedures, but also accurate folding around disulphide bonds is known to occur readily in the endoplasmic reticulum (ER). Correct folding is particularly vital in the expression of an active enzyme such as AChE. Smith et al, (1985) found that recovery of correctly folded, fully active prochymosin was only possible when secreted rather than intracellularly expressed from *S. cerevisiae*. Attempts to produce human serum albumin (HSA) intracellularly in *S. cerevisiae* also



resulted in insoluble and inactive product which was subsequently found to have the correct conformation when directed through the secretory pathway (Echeverry et al, 1986). This human protein of 65 KDa has no potential sites of N-linked glycosylation (1.5.6.b) but has 17 disulphide bonds as an integral part of its native conformation and clearly is only processed correctly by yeast on passing through the secretory pathway.

N-terminal manipulations can be performed on intracellularly expressed proteins if required, either to avoid ubiquitin dependent degradation, or because the N-terminus is essential for activity or may give rise to immunogenicity problems in the case of therapeutic proteins. For example removal of an unwanted methionine at the N-terminus can be achieved by cyanogen bromide cleavage or by using the *E. coli* methionine amino peptidase enzyme, or more elegantly the desired N-terminus can be achieved *in vivo* by engineering of a fusion protein with ubiquitin (1.5.5.e). However, successful direction of the desired protein through the secretory pathway either by its natural secretion signal or by use of another heterologous signal or a native yeast signal (1.5.7) results in proper N-terminal processing by virtue of signal peptidase cleavage (1.5.6.a). This can also be achieved at a later point in the secretory pathway, if desired, by use of a peptide prosequence, such as that found in the natively secreted  $\alpha$ -factor (1.5.6.b).

It should be noted that the level of secretion achieved varies widely and empirically depends on the choice of secretion signal, the nature of the protein to be expressed (generally, peptides and small proteins are secreted fairly efficiently, but with larger proteins secretion is unpredictable, depending on factors such as hydrophobicity) and the strain of *S. cerevisiae* selected. DAcHE is a naturally secreted protein of medium size and contains

hydrophobic sequences. It was entirely possible that it may have been secreted by virtue of its native signal peptide. Alternatively it may not have been efficiently secreted even with a *S. cerevisiae* secretion signal attached.

Increased recovery of secreted heterologous proteins may also be a bonus. Secretion of human growth hormone led to as much as a 300 fold increase in recovery (Brake et al, 1984) but it should also be noted that secretion levels are often lower than when foreign proteins are expressed intracellularly and that a fully active, correctly folded product may be produced in the cytoplasm. Expression levels of secreted proteins can be subject to limitations, for example a 'saturation effect' whereby high levels of heterologous mRNA are not realised in the amounts of secreted product. Intracellular accumulation of precursor forms can occur leading to an inhibition of secretion. The secretory machinery effectively becomes 'clogged' and this can occur particularly when the heterologous gene is at multiple copy number (Loison, 1994).

Recombinant human  $\alpha$ -antitrypsin is an example of the sometimes quixotic nature of secretion in *S. cerevisiae*. High levels of soluble active enzyme accumulate in the cytoplasm but secretion is problematic (Cabezon et al, 1984; Moir and Davidow, 1991). This is interesting because  $\alpha$ -antitrypsin is a naturally secreted protein and generally might be expected to not only be easily secreted but to require the conducive environment of the ER for correct folding to take place.

#### 1.5.6.a. The Yeast Secretory Pathway

The secretory pathway was defined in *S. cerevisiae* with the help of a series of secretion defective mutants (sec mutants) (Novick et al, 1980). The secretory pathway was



shown to closely resemble that of higher eukaryotes and proteins destined for the pathway to contain 'signal' peptides at the N-terminal end (Milstein et al, 1972; Schechter et al, 1975).

Blobel and Dobberstein's signal hypothesis (1975) stated that the signal peptide, following translation, is bound by receptors in the ER membrane leading to translocation. Proteins interacting with the signal peptide include the signal recognition particle (Srp) and the Srp receptor first discovered in mammalian cells (Seigel and Walter, 1988) and similar proteins sharing homology have been discovered in yeast (Hann et al, 1989). It is thought that the Srp binds the signal peptide following translation and that subsequent interaction with the Srp receptor in the ER membrane and association with other proteins leads to translocation.

Following translocation the signal peptide is cleaved away by a signal peptidase, releasing the mature protein into the ER lumen. Proteins are translocated in an unfolded state and the maintenance of this state depends on the binding of molecular chaperones. In *E. coli* it has been demonstrated that a factor termed SecB is involved in maintaining the unfolded state of the pre form of maltose binding protein (MBP) (Collier et al, 1988). SecB- strains of *E. coli* exhibit minimal export of MBP and furthermore the protein isolated from SecB- strains has been shown to consist of a highly folded protein that is resistant to protease digestion (Kumamoto et al, 1988). In eukaryotic cells Srp may perform a similar function. Crooke et al (1988) demonstrated that Srp could facilitate the translocation of denatured prepro  $\alpha$ -factor into *S. cerevisiae* microsomes. The fact that denaturation was required suggests that Srp is not able to actively unfold proteins during processing, but rather binds immediately following translation thereby maintaining an unfolded

state.

Although the importance of the Srp has long been recognised there is recent evidence that in yeast there may be two routes of translocation across the ER membrane for secretory proteins one of which is dependent on the Srp and the other independent. Rothe and Lehle (1998) designed mutated invertase signal peptides with either a Pro or Gly residue or only four hydrophobic residues in the hydrophobic core that rendered them incapable of interacting with the Srp. However, subsequent expression of the mutant variants in an Srp deficient strain of *S. cerevisiae* demonstrated that translocation of invertase was not impaired, despite the fact that wild type invertase is known to be translocated through interaction with the Srp. Protein targeting to the ER can therefore occur in *S. cerevisiae* independently of the Srp.

In yeast, members of the heat shock protein family may also be involved in translocation (Chirico et al, 1988). It has been shown, for example, that *ssa1* mutants (defective in the heat shock protein SSA1) accumulate and fail to translocate unprocessed precursors of the homologous proteins carboxypeptidase Y and  $\alpha$ -factor (Werner-Washburner et al, 1987).

The composition of the signal peptide is crucial to effective translocation across the ER membrane. Interestingly, although the function of signal peptides is widely conserved, comparative amino acid sequences from both prokaryotes and eukaryotes reveals that no real consensus sequence exists (Vov Heijne, 1984; Perlman and Halvorsen, 1983). However, all signal peptides described to date consist of three distinct domains: positively charged N-terminal domain, a central hydrophobic core, and a polar caboxyl terminus containing the site of cleavage. The individual net charge of the three domains is what



appears to be conserved rather than sequence or length (Von Heijne, 1985). However, the sequence surrounding the cleavage site at the C-terminal end is fairly conserved. For example Ala is the favoured residue at the adjacent site to the actual cleavage site (position -1) (Von Heijne, 1983). Downstream of the hydrophobic core, more than one potential site for cleavage is thought to exist and these sites are thought to compete for binding of the signal peptidase, binding most frequently occurring at the preferred site. Disruption of cleavage sites in the C-terminal domain can result in the precursor form of a protein being translocated to the ER. For example Schauer *et al* (1985) showed that replacement of the favoured Ala residue at -1 with a Val residue in the signal peptide of invertase resulted in translocation of the unprocessed form.

The hydrophobic core of the signal peptide is important for translocation since it interacts directly with the SrP and ER membrane (Wickner *et al*, 1980; Engleman, 1981). Radical disruption of the hydrophobic core can effectively abolish secretion. For example, deletion of 4 amino acids from the hydrophobic domain of the invertase signal peptide led to accumulation of invertase in the cytoplasm and the enzyme was shown to have no interaction with the ER (Perlman *et al*, 1986). Human lysozyme was efficiently secreted by *S. cerevisiae* when fused to a signal peptide optimized for hydrophobicity (Yamamoto and Kikuchi, 1989).

The positively charged N-terminal domain of signal peptides is thought to greatly enhance the efficiency of translocation by facilitating the interaction between the signal peptide and molecular chaperones involved in the translocation process. In *E. coli* it has been demonstrated that positive charges in signal peptides do indeed interact with proteins such as the SecA protein a component of the *E. coli* secretory pathway essential for translocation

(Akita et al, 1990). Further, experiments with randomly generated signal sequences have suggested that a positively charged N-terminal domain is important for function in *E. coli* (Zhang and Broome-Smith, 1989). However, it should be noted that it is not an absolute requirement since elimination of the three positively charged amino acids of the N-terminal domain of the MBP signal peptide were shown to have no effect on secretion (Puziss et al, 1989). In contrast, unlike *E. coli* native signal peptides, not all *S. cerevisiae* signal peptides contain a positively charged N-terminal domain and random sequences without a net positive charge in the N-terminal domain have been shown to be functional in yeast (Kaiser and Botstein, 1986; Kaiser, 1987). This again suggests the relative flexibility of signal sequences involved in targeting proteins to the ER in *S. cerevisiae*. Interestingly, although a net positive charge is far from essential, misplacement of a positive charge may have a significant effect. Green et al (1989) showed that replacement of the positively charged Arg residue with a neutral Phe in the N-terminal domain pre region of the  $\alpha$ -factor signal peptide had no demonstrable effect on secretion but that alteration by only one position of the Arg residue resulted in a significant reduction in secretion. Recently, secretion of a fusion protein between the prepro- $\alpha$ -factor and somatostatin in yeast was shown to be severely disrupted by alteration of the three N-terminal amino acids of the native prepro- $\alpha$ -factor (Met-Arg-Phe) to Met-Phe-Lys. Significant amounts of unglycosylated precursor molecule were shown to accumulate intracellularly indicating disruption of translocation to the ER (Lee et al, 1996). It would appear if the work of Green et al (1989) is taken into account that the disruption is due to the misplacement of the positive charge (Lys) rather than to the replacement of Arg with Phe.

In addition to molecular chaperones involved in



translocation, resident proteins within the ER are known to be involved in the refolding, solubilisation, and further transportation of secretory proteins. Two proteins known to be involved are binding protein (BiP) (Rose et al, 1989; Normington et al, 1989) and protein disulphide isomerase (PDI) (Hardwick et al, 1990). Mutations in the two active sites of the thiodoxin-like domains of PDI, for example, have been correlated with a reduced rate of folding of CpY *in vivo* (Holst et al, 1997). Reduction of BiP has been shown to decrease heterologous protein secretion in *S. cerevisiae*. Experiments with a *S. cerevisiae* strain in which BiP expression levels could be manipulated have demonstrated that expression levels of BiP lower than wild type were correlated with decreased secretion levels of human granulocyte colony stimulating factor, bovine pancreatic trypsin inhibitor, and *Schizosaccharomyces pombe* acid phosphatase from *S. cerevisiae*, providing further evidence for a positive role for BiP in secretion. However, expression levels of BiP higher than wild type did not correlate with either increased or decreased secretion levels (Robinson et al, 1996). This is interesting because overexpression of BiP might have been predicted to improve secretion by counteracting the limiting effects of depletion during the prolonged overexpression of secreted heterologous proteins (levels of both BiP and PDI have been shown to be significantly reduced in such cases (Robinson and Wittrup, 1995)).

Following refolding and preliminary glycosylation in the ER (see 1.5.6.b) proteins can then be routed to the cis face of the Golgi apparatus via ER derived membrane vesicles where further modifications take place. Other post-translational modifications such as the attachment of glycoposphatidylinositol (GPI) anchors are known to take place in the ER prior to transfer to the Golgi where a postulated transamidase removes a C-terminal peptide from the future anchor containing protein and attaches the

anchor (Benghezal et al, 1996). The native DAcE contains such an anchor and presumably attachment of an anchor by *S. cerevisiae* will depend on the DAcE being translocated to the ER. Outer core glycosylation takes place in the Golgi (1.5.6.b) and also final mannose side chain attachments to such modifications as GPI anchors (Sipos et al, 1995). In the Golgi modified proteins are sorted and can be routed to the correct destination: back to the ER, to other subcellular locations, or transported for secretion from the cell (Novick et al, 1981; Schekman, 1982). Overexpression of two proteins involved in the targeting and fusion of Golgi derived secretory vesicles to the plasma membrane, Sso1 and Sso2, has been shown to enhance the secretion of *Bacillus*  $\alpha$ -amylase from *S. cerevisiae* by approximately 5 fold (Ruohonen et al, 1997).

#### 1.5.6.b. Glycosylation

In addition to refolding in the ER the major modification made to proteins routed through the secretory pathway is glycosylation. As in other eukaryotes both N-linked and O-linked glycosylation occurs. The structure of the core oligosaccharide that is attached to Asn residues of proteins passing through the secretory pathway is identical to that found in mammalian glycoproteins but there is a major difference in subsequent modifications made to the core. Mammalian glycoproteins undergo extensive 'trimming' of the core followed by the addition of sialic acid, galactose, or N-acetylglucosamine (GNAC). In contrast homologous yeast glycoproteins undergo a less extensive trimming followed by considerable elongation of the core with outer chains of mannose residues (Shekman, 1985). This may also occur during the processing of heterologous proteins by yeast which can lead to inactivity or antigenicity problems with the final product (Brake, 1990).

The sugar content of a heterologous protein processed in



yeast may therefore be grossly different not only in terms of overall sugar content but also in terms of the nature of the sugars attached. The mannose content is likely to be far higher and the other sugars such as sialic acid added to mammalian proteins following trimming of the core are likely to be absent. The difference in yeast and mammalian glycosylation processing is well illustrated by the heterologous expression of the human immunodeficiency virus (HIV) envelope glycoprotein gp120. When a portion of the protein containing 24 potential sites of N-linked glycosylation is expressed in *S. cerevisiae* a hyperglycosylated form of approximately 600 KDa is produced compared to a glycosylated product of 120 KDa when expressed in mammalian cells (Hitzeman et al, 1990). The hyperglycosylation of gp120 when expressed in yeast affects its ability to bind the CD4 receptor (Smith et al, 1987). Human  $\alpha$ -1-antitrypsin, which only contains 3 potential sites of N-linked glycosylation, is also hyperglycosylated when secreted from wild type *S. cerevisiae* (Moir and Dumais, 1987; Kang et al, 1996). When human pancreatic ribonuclease was recently expressed in *S. cerevisiae* it was found to be hyperglycosylated (Ribo et al, 1996).

Outer chain glycosylation mutants such as the *mnn9* mutant (Tsai et al, 1984) (see below) can be used to alleviate this problem. Secretion of human  $\alpha$ -1-antitrypsin using the *mnn9* mutant results in a protein which is not hyperglycosylated but which is slightly smaller than the native protein suggesting differences between the native and heterologous protein in terms of inner core glycosylation (Moir and Dumais, 1987). Expression of gp120 in the *mnn9* mutant results in a protein of comparable size to that produced in mammalian cells (Hitzeman et al, 1990).

O-linked attachment of short manooligosaccharide chains of one to four units occurs in yeast to both homologous and heterologous proteins at Ser and Thr residues. O-

glycosylation of secreted heterologous proteins has been observed to occur at the same sites as in the native protein (Ernst et al, 1987; Van Arsdel et al, 1987). Two O-mannosyltransferases thought to act as a complex encoded by the genes *pmt1p* and *pmt2p* are known to catalyse O-linked glycosylation of proteins in the yeast ER (Gentzsch et al, 1995).

N-linked core glycosylation in eukaryotes occurs in the ER. It should be mentioned at this point that the relationship between protein folding and glycosylation is complex. There is evidence to suggest that core glycosylation takes place prior to the final steps in protein folding since inhibition of glycosylation at certain sites in particular glycoproteins is known to reduce the rate of folding (Riderer and Hinnen, 1990; Winther et al, 1991). Recent experimental evidence where new N-linked glycosylation sites were introduced into yeast carboxypeptidase Y showed that the level of new glycosylation that occurred was related to the conditions for folding, thus revealing that glycosylation does not necessarily occur prior to folding and that 'competition' between folding and glycosylation reactions may occur in the ER (Holst et al, 1996). The core unit consists of 14 sugar residues (3 glucose, 9 mannose, and 2 GNAC and the attachment occurs at Asn residues within the sequence Asn-X-Ser/Thr where X can be any amino acid except proline (Ballou, 1982; Lehle and Bause, 1984). The core oligosaccharide is assembled on a phosphorylated dolichol glycolipid in the membrane of the ER and in yeast the first step involves the attachment of the GNAC residue by a specific transferase encoded by the gene *ALG7* (Rine et al, 1983). This is the step inhibited by the antibiotic tunicamycin (Lehle and Tanner, 1976). The second GNAC residue is then added before the attachment of the first mannose residue by the action of  $\beta$ -1,4 mannosyltransferase. The additional mannose residues are then added and the final step involves the addition of the



three glucose residues to the core. In yeast the isolation of *alg* (Asn linked glycosylation) mutants based on selection by [<sup>3</sup>H] mannose killing has allowed detailed characterisation of the individual transfer reactions involved (Huffaker et al, 1982; Huffaker and Robbins, 1983; Runge et al, 1984; Runge and Robbins, 1986).

An oligosaccharide transferase consisting of multiple subunits is responsible for the transfer of the completed core unit to the protein in the lumen of the ER and attachment to the Asn residue by means of a N-glycosidic bond (Kaplan et al, 1987). It is known that six loci at least (*STT3*, *OST1*, *WBP1*, *OST3*, *SWP1*, and *OST2*) are involved in coding for components of the transferase complex and recently a seventh *OST5* was identified, deletion of which results in reduced activity of the complex (Zufferey et al, 1995; Reiss et al, 1997; Spirig et al, 1997). It is at this point that the major difference between glycosylation of mammalian proteins and yeast proteins occurs. As previously mentioned trimming of the core in mammalian cells leaves only three mannose residues remaining which are then modified by the addition of sialic acid, GNAC, or galactose as required (Kornfield and Kornfield, 1985). In yeast the core oligosaccharide undergoes further elongation following transfer to the Golgi.

The isolation of *mnn* (mannan defective) mutants has been crucial to the elucidation of the outer chain 'flowering' of mannose residues built upon the oligosaccharide core that takes place in the Golgi. The *mnn9* mutant, for example, lacks the active mannosyltransferase responsible for initiating outer chain glycosylation (Tsai et al, 1984). The initiation point for elongation suggested by Hernandez et al (1989) is the  $\alpha$ -1,3 mannose as shown in Figure 1.5. Elongation is then achieved by addition of further outer chains (as many as 15). Other *mnn* mutants have helped to define the structure of the side branches of

the oligosaccharide core and outer chain extensions. For example the *mnn1* mutant lacks all terminal  $\alpha$ -1,3 mannoses attached to  $\alpha$ -1,2 linked mannoses in the core, outer chain, and also on O-linked oligosaccharides. In the *mnn2* mutant the mannose backbone of the outer chain is synthesised but the flowering side chains are lost.

Glycosylation of homologous yeast proteins affects further protein processing and passage through the secretory pathway. It has been shown that secretion of invertase, acid phosphatase, and  $\alpha$ -galactosidase, into the periplasmic space is related to glycosylation. Secreted invertase, for example, exists mainly in an octomeric form. Octomers are not formed, however, when cells are grown in the presence of tunicamycin and subsequent secretion is inhibited (Esmon *et al*, 1987).

Evidence from studies of the secreted  $\alpha$ -factor (a 13 residue peptide required for efficient mating with the a mating type of haploid *S. cerevisiae* cells) show that glycosylation is related to protein processing. The  $\alpha$ -factor is processed from larger glycosylated precursors (Brake *et al*, 1983; Julius *et al*, 1984) encoded by the genes *MF $\alpha$ 1* and *MF $\alpha$ 2* (Kurjan and Herskowitz, 1982; Singh *et al*, 1983). The translated product of the major *MF $\alpha$ 1* gene consists of 165 amino acid residues containing four repeats of the mature  $\alpha$ -factor peptide each of which is separated by a spacer sequence of 6-8 residues. The 'prepro' region consists of 83 amino acids including the hydrophobic signal peptide sequence and three potential sites for Asn-linked glycosylation. Cleavage of the pre signal peptide by a signal peptidase following translocation to the ER occurs between residues 19 and 20 of the precursor (Waters *et al*, 1988). The pro- $\alpha$ -factor is then core glycosylated in the ER before translocation to the Golgi. Interestingly, incomplete and unglycosylated forms of the precursor have

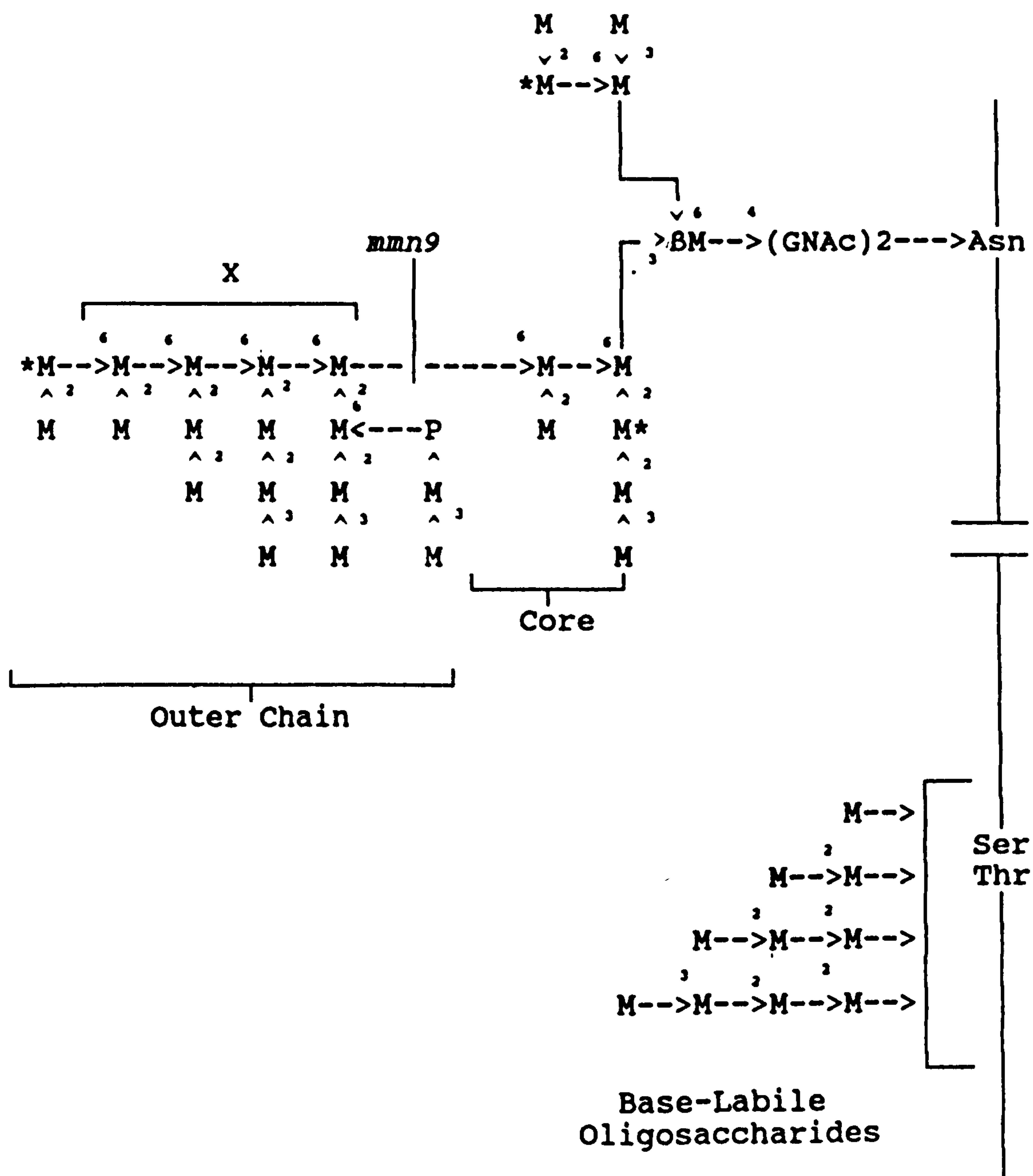


Figure 1.5. N-Linked Glycosylation in *S. cerevisiae*. The  $\alpha$  mannose linkages are illustrated. The *mnn9* mutant lacks the specific mannosyltransferase necessary for initiation of the outer core (after Ballou, 1990).

Wild type: X = 10 - 15

*mnn10*: X = 1 - 4

\*: Additional phosphorylation sites.





been shown to accumulate in the ER in *sec* mutants suggesting, as mentioned previously, that core glycosylation of proteins in the secretory pathway is related to subsequent translocation (Julius et al, 1984). The glycosylated proprecursor is cleaved in the Golgi by the action of a membrane bound calcium dependent endoprotease encoded by the *KEX2* gene (Fuller et al, 1989), cleavage occurring on the carboxyl side of the Lys-Arg sequence in the spacer. This cleavage has been shown to be blocked in *kex2* mutants and to be associated with the secretion of a hyperglycosylated form of the pro- $\alpha$ -factor (Julius et al, 1984).

Final maturation of the fully active  $\alpha$ -factor depends on further processing at the N and C terminal ends of the peptide. Glu-Ala and Asp-Ala dipeptides are removed from the N-terminus by the action of the *STE13* gene product, a dipeptidylaminopeptidase (Julius et al, 1983). The C-terminus is also 'trimmed' by removal of the Arg and Lys residues. A serine protease with carboxypeptidase B-like activity encoded by the *KEX1* gene has been shown to carry out this process (Dmochowska et al, 1987).

In certain secretory proteins such as the  $\alpha$ -factor and carboxypeptidase Y (CpY) the C-terminus of the pro region may have a chaperone like function that is important for correct folding and subsequent transport from the ER. Investigation of different substitutions into this region of the pro-sequence of CpY by Chaudhuri et al (1995) revealed that replacement of Leu<sup>108</sup> at the C-terminus with a polar residue such as Lys or Arg resulted in poor levels of expression and retention in the ER. An induction in the levels of BiP was also noted when these mutant proteins were expressed suggesting that misfolding of CpY had occurred (Chaudhuri et al, 1995).

#### 1.5.6.c. Secretion Signals for Heterologous Expression

Heterologous gene products have been successfully secreted from *S. cerevisiae* using their natural secretion signals demonstrating that in some instances the 'foreign' signal peptide is recognised and processed by *S. cerevisiae* and is successfully directed through the secretory pathway. For example the unmodified cDNAs of the leukocyte interferons (IFN), IFN- $\alpha$ 1 and IFN- $\alpha$ 2 are sufficient for successful secretion (Hitzeman et al, 1983). However, closer examination of signal peptide processing by *S. cerevisiae* in these cases suggested that there is a difference in signal recognition between yeast and mammalian cells since 36% of IFN- $\alpha$ 1 and 47% of IFN- $\alpha$ 2 secreted into the media was shown to be misprocessed at the signal peptide level. The misprocessed protein was shown to have been cleaved by the yeast signal peptidase between the Cys and Ser residues at positions -4 and -3 upstream of the mature protein rather than at the correct position between the Gly and Cys residues at positions -1 and +1. Furthermore, in the case of IFN- $\alpha$ 1, 8% of the secreted protein was misprocessed further upstream in the signal peptide. Approximately 50% of the expressed interferon was not secreted and remained cell associated (Hitzeman et al, 1983).

The differences existing in signal recognition between yeast and mammalian cells was also highlighted when human growth hormone containing its native signal peptide was expressed in yeast (Goeddel et al, 1979). Approximately 10% of the protein produced is correctly processed and secreted into the media but 90% is not processed at all, retaining the complete signal sequence, and remaining associated with the cell (Hitzeman et al, 1984).

In contrast the native secretion signal of HSA has been shown to be efficient in yeast with around 50% of the protein secreted into the media and correct cleavage at the



native prosequence site (Etcheverry et al, 1986). Interestingly, the prosequence can be cleaved *in vitro* by an extract of wild type *S. cerevisiae* but not by an extract of a *kex2*- mutant strain strongly suggesting the involvement of the KEX2 endoprotease in prosequence cleavage; the only difference being that the amide bond is cleaved following an Arg-Arg amino acid pairing rather than the Lys-Arg pairing observed in yeast.

The HSA secretion signal can also be used for the secretion of other heterologous proteins. Lasky et al, (1986) used the prepro sequence of HSA to successfully secrete the gp120 protein of HIV which was correctly processed at the Arg-Arg prosequence site. High level secretion of human  $\alpha$ 1-antitrypsin has been achieved in *S. cerevisiae* using the inulinase secretion signal from another yeast *Kluveromyces marxianus*. N-terminal sequencing of the purified recombinant protein revealed that it had been correctly processed at the KEX2 cleavage site (Kang et al, 1996). Thus a heterologous signal sequence from one foreign protein can be employed to secrete a different heterologous protein in *S. cerevisiae*. In general, however, the most successfully employed secretion signals used for heterologous expression have been homologous yeast signals attached to mature heterologous protein sequences. The most commonly employed homologous secretion signals are those of the  $\alpha$ -factor, killer toxin, invertase, and acid phosphatase genes. For example, the yeast invertase signal has been used for the secretion of properly processed human IFN- $\alpha$ 2 (Chang et al, 1986). Correct N-terminal processing of the heterologous product can be accomplished by splicing of the signal sequence with the signal peptidase cleavage site adjacent to the first amino acid of the mature heterologous sequence (Hitzeman et al, 1990). It has been found that yeast appears to be tolerant of amino acids to the right of the cleavage site and that alterations at this point still results in correct processing by the signal



peptidase in the majority of cases (Chang et al, 1986). It should be stressed that since exact in frame fusions are required for production of the required secreted product considerable care should be taken in the design of subcloning strategies that include synthetic DNA linkers for the convenient splicing of heterologous genes with the homologous or heterologous secretion signal to be used. This may, for example, include design of a linker that contains the KEX2 cleavage site so that splicing at a restriction recognition sequence upstream of the site in the  $\alpha$ -prosequence directly joins the heterologous gene to the prosequence in frame with the mature sequence beginning at the next amino acid position to the right of a Lys-Arg processing site. This was the strategy adopted in the current study for subcloning the DACH<sub>E</sub> gene into the *P. pastoris* vector pPIC9 in frame with the  $\alpha$ -factor leader (4.2.3). Alternatively, unwanted DNA sequences generated between the secretion signal and the mature heterologous sequence during subcloning may be removed by site-directed mutagenesis (Hitzeman et al, 1990).

The most frequently used secretion signal is probably that of the signal sequence of the  $\alpha$ -factor of *S. cerevisiae* (1.5.6.b). This signal has been used for the successful secretion of a wide variety of heterologous proteins such as hEGF (Brake et al, 1984),  $\beta$ -endorphin (Bitter et al, 1984), and IFN- $\alpha$ 1 (Singh et al, 1984). The secretion of hEGF using the  $\alpha$ -factor signal was particularly successful with a concomitant increase in expression levels from  $\approx 30\mu\text{g/L}$  when expressed intracellularly to more than  $1\text{mg/L}$  when secreted. In all of the cases mentioned correct processing of the prosequence occurred with KEX2 protease cleavage to the right of the Lys-Arg consensus pairing with release of the mature protein. However, these secreted heterologous proteins were found to retain the Glu-Ala spacer sequence found in the natural pro- $\alpha$ -factor at the N-terminus. The



cause has been suggested to be due to limiting amounts of the *STE13* encoded dipeptidylaminopeptidase (1.5.6.b) when these heterologous genes are overexpressed (Brake, 1990).

In the case of hEGF site-directed mutagenesis of the fusion sequence was sufficient to circumvent this problem by deleting the spacer segment. The resultant fusion placed the mature protein sequence directly adjacent to the Lys-Arg cleavage site and secretion was observed at similar levels to constructs containing the spacer sequence (Brake et al, 1985). However, this is not always the case since  $\alpha$ -factor fusions without the spacer sequence have sometimes been observed to result in intracellular accumulation of product or to result in the secretion of misprocessed or partially processed proteins. This in direct contrast to correct processing and secretion when the spacer segment is present. These misprocessed forms can also be hyperglycosylated in a similar manner to that observed for the hyperglycosylated pro- $\alpha$ -factor peptides produced in *kex2* mutants (1.5.6.b). This has been shown to occur to > 50% of the IFN- $\alpha$ 1 product expressed from  $\alpha$ -factor fusions lacking spacer peptide sequences. Inclusion of a (Glu-Ala)<sub>2</sub> spacer results in correct processing (Zsebo et al, 1986). When the  $\alpha$ -factor leader was used to secrete a precursor of insulin in *S. cerevisiae* without the spacer a significant proportion of the recombinant protein was unprocessed at the prosequence level and hyperglycosylated. Introduction of a spacer peptide after the KEX2 cleavage site resulted in greatly enhanced processing of the insulin precursor (Kjeldsen et al, 1996). Thus, in some cases at least, processing by the *KEX2* gene product must be affected by sequences adjacent to the processing site.

If a Glu-Ala spacer is deemed to be beneficial for efficient secretion using the  $\alpha$ -factor leader then increased expression of the limiting *STE13* product may complete processing of overexpressed heterologous products

at the N-terminus (Brake, 1990). Alternatively, increased expression of KEX2 may improve processing of fusion precursors in the absence of a spacer sequence. When transforming growth factor (TGF)- $\alpha$ 1 is secreted using the  $\alpha$ -factor leader both correctly processed and uncleaved forms exhibiting varying degrees of glycosylation are produced. Overexpression of KEX2 by insertion of the gene into the high copy number plasmid carrying the  $\alpha$ -factor-TGF- $\alpha$ 1 fusion successfully resulted in the higher level secretion of only the correctly processed form (Barr et al, 1987).

More recently the  $\alpha$ -factor leader has been used for the secretion of human insulin-like growth factor I. The protein released into the culture medium was found to be correctly processed but some unprocessed forms were retained intracellularly (Steube et al, 1991). It has also been used for the successful secretion to the culture medium of human fibrinogen which was found to be correctly processed (Roy et al, 1995).

#### 1.5.7. An Alternative Yeast Expression System: *Pichia pastoris*

In recent years alternative yeasts to *S. cerevisiae* have been investigated in an attempt to build upon and improve the performance of yeast expression systems. *Pichia pastoris*, a methylotrophic yeast, which can be cultured to high cell densities ( $\approx 130$ g dry wt cells/L (Wegner, 1983)), has proven itself an excellent alternative to *S. cerevisiae* in the production of heterologous proteins. Foreign proteins are often expressed at g/L levels and expression using a *Pichia* yeast system may be preferable not only in terms of levels of production for particular proteins but also for other reasons. For example, glycosylation patterns of proteins differ between *P. pastoris* and *S. cerevisiae* a product which is hyperglycosylated by *S.*



*cerevisiae* may not be by *P. pastoris*.

In common with other methylotrophic yeasts such as *Hansuela polymorpha* and *Candida albicans*, which have also been used for heterologous expression, *P. pastoris* can be cultured using methanol as the sole carbon source. These yeasts share a common methanol utilization pathway. The methanol inducible alcohol oxidase, which is the first component of the pathway, has been found to account for as much as 30% of the total protein in *P. pastoris* cells grown on methanol but is undetectable in cells grown on glucose, glycerol, or ethanol (Wegner and Harder, 1986). It is the promoter of this tightly regulated gene that has been used to construct integrative vectors for heterologous gene expression in *P. pastoris*. Following catabolism of the methanol substrate to formaldehyde (Anthony, 1982) the pathway forks such that the formaldehyde product can either be assimilated or oxidized by two dehydrogenase reactions to carbon dioxide (Gellissen et al, 1994)

*Pichia* systems typically use integrative vectors for several reasons which include the general advantages of high stability, the opportunity of constructing multicopy strains, direction of integration to specific loci, and the possibility of using alternative modes of integration (with or without transplacement of the methanol inducible alcohol oxidase gene *AOX1*). Furthermore, studies using autonomously replicating vectors which include a segment of *P. pastoris* ARS (usually *PARS1*) have shown that such vectors tend to be unstable, of low copy number, and very often integrate unpredictably into the host cell genome at the site of a region of homology (*AOX1*, *PARS1*, or the selectable marker, typically *HIS4*). An example of the latter type of ARS vector is pHIL-A1 (Sreekrishna et al, 1987). It has been noted, however, by Sreekrishna et al (1987) that ARS plasmids have the useful advantage of high transformation efficiencies of *P. pastoris* compared to

linearized integrative vectors.

#### 1.5.7.a. *P. pastoris* Expression Cassettes

The typical integrative expression cassettes that have been used with different degrees of success for heterologous protein production (Romanos *et al*, 1992; Sreekrishna, 1993; Sreekrishna and Krop, 1996) include in one half the usual *E. coli* sequences from pBR322 or pUC ( $AMP^R$  and ColE1 ori) enabling subcloning manipulations to be carried out in *E. coli* and in the other half of the vector the sequences required for integration, selection, and expression in *P. pastoris*. These two halves can be cleaved by digestion at two opposite restriction sites such that the linear yeast sequences can be transformed and targeted to a specific locus in the *P. pastoris* genome.

The gene to be expressed is subcloned into a vector at a suitable restriction site between 5' and 3' flanking sequences of AOX1. The yeast selectable marker which is invariably *HIS4* (coding for histidinol dehydrogenase and complementing the defective *his4* gene in an auxotrophic *P. pastoris* strain such as GS115 or SMD1168) resides downstream of the heterologous gene and upstream of the 3' AOX1 flanking sequence. There is a restriction site such as *Bgl*III at the start of the 5' AOX1 sequence and one immediately following the 3' AOX1 sequence. Digestion of the vector with *Bgl*III thus results in a linear fragment ready to transform into *P. pastoris*. The ends of the linear fragment are homologous with the 5' and 3' ends of the natural AOX1 locus and a double recombination event can result in the replacement of AOX1 with the heterologous gene (Cregg *et al*, 1987; Sreekrishna *et al* 1988b; Sreekrishna, 1993).

The site specific eviction of AOX1 has been estimated to occur at a frequency of  $\approx 1-5/20$  His<sup>+</sup> transformants.



Following an initial selection using the auxotrophic marker His<sup>+</sup> transformants are grown on minimal methanol medium where clones deleted for the structural AOX1 gene can be identified by a slow growth characteristic. Non-AOX1 deleted His<sup>+</sup> clones exhibit normal growth on minimal methanol medium due to functional methanol inducible alcohol oxidase. The reason *P. pastoris* colonies deleted for AOX1 are able to grow at all on minimal methanol medium is due to the presence of a second methanol inducible alcohol oxidase locus (AOX2) from which the enzyme is expressed at low level (Cregg and Madden, 1987). pPIC9 (Scorer et al, 1994) commercially available from Invitrogen is a typical example of this type of vector (Figure 2.4). Other examples include pHIL-D2 and pHIL-D7 (Sreekrishna et al, 1997).

#### 1.5.7.b. Heterologous Protein Secretion from *P. pastoris*

*Pichia* systems can be used for intracellular expression and in such cases it has been noted that it is preferable to use AOX1 deleted cells because they contain only low levels of alcohol oxidase when induced on methanol making purification of the heterologous protein easier (Sreekrishna et al, 1997). However, it is often preferable to secrete the protein for reasons that have already been discussed for *S. cerevisiae* (1.5.7).

Successful secretion of a heterologous protein from *P. pastoris* is, like secretion from *S. cerevisiae*, largely unpredictable. In certain instances it has been found that the native signal peptide is sufficient for secretion of the heterologous protein, for example invertase (Sreekrishna et al, 1987) and bovine lysozyme (Digan et al, 1989), but in general a suitable yeast signal peptide is necessary. The commercially available pPIC9 vector (Invitrogen) contains the  $\alpha$ -factor secretion signal from *S. cerevisiae* which must be fused in frame with the gene to be

expressed. The  $\alpha$ -factor signal, in combination with the KEX2 cleavage site (1.5.6.b), has been successfully used for the secretion of heterologous gene products from *P. pastoris*. This vector has been recently employed to secrete two vertebrate AChEs from *P. pastoris* but it was found that levels of secretion using the  $\alpha$ -factor leader were lower than those obtained with the native signal peptides (Morel and Massoulie, 1997). Expression levels in general of both rat and *Bungarus* (snake venom) AChE were found to be considerably lower (500-1000X) than that of certain other heterologous proteins that have been successfully expressed in *P. pastoris* such as bovine lysozyme (Digan et al, 1989); but it is perhaps not surprising that the secretion of smaller heterologous proteins has been more successful. Examples include aprotinin (Wagner et al, 1992), thrombomodulin fragment (White et al, 1994), blood factor XII (White et al, 1994), a fragment of amyloid  $\beta$ -protein (Van Nostrund et al, 1994), and antibody single chain Fv fragment (Ridder et al, 1995).

Alternative signal peptides have been used, for example the *P. pastoris* PHO1 (acid phosphatase) secretion signal has been used in conjunction with a KEX2 cleavage site by Laroche et al (1994) for the high level secretion of a tick anticoagulant peptide (up to 1.7g/L). The PHO1 signal peptide was also used for expression of vertebrate AChE in *P. pastoris* by Morel and Massoulie (1997) but again levels of secretion were found to be lower than those obtained using the native signal peptides.

Recently it has been suggested that over-expression of ubiquitin may enhance heterologous protein secretion from *P. pastoris* since it is now known that ubiquitin is a normally secreted component of this yeast and may have a role to play in protein secretion (Sreekrishna et al, 1995). Overexpression of ubiquitin has been shown to enhance secretion of a heterologous protein (human



leukocyte protease inhibitor) in *S. cerevisiae* by Chen et al (1994). It seems likely that a ubiquitin overexpressing strain may improve heterologous protein secretion from *P. pastoris* still further.

#### 1.5.7.c. Factors Affecting Optimal Expression in *P. pastoris*

The factors which affect heterologous expression in *S. cerevisiae* have also been shown to affect the efficiency of expression in *P. pastoris*. They include gene dosage, promoter and transcriptional terminator design (efficiency of transcription and stability of mRNA), AUG start codon context (efficiency of translation), and protein stability, all of which have already been reviewed in the context of *S. cerevisiae* (1.5.5).

It has been noted in *P. pastoris* for example that a significant increase in production is seen with certain heterologous proteins when multiple copies (>10) of the gene are present. This has been the case with the expression of tetanus toxic fragment C (Clare et al, 1991a), *Bordetella pertussis* pertactin P69 (Romanos et al, 1991), and mouse epidermal growth factor (Clare et al, 1991b). Multiple tandem integration of the expression cassette occurs naturally during the spheroplast method of transformation and transformants tend to display a wide range of copy numbers (Clare et al, 1991a and b; Sreekrishna et al, 1993). Individual clones can then be analysed for maximum production. An alternative approach is to design multiple copy number vectors or to use DNA concatamers to achieve multiple intergration (Sreekrishna and Kropp, 1996). The former approach is preferable, however, since a single copy may result in optimal production. This has proved to be the case when hepatitis B surface antigen and HSA were expressed in *P. pastoris* (Cregg et al, 1987; Sreekrishna et al, 1990). Increasing

gene dosage was found not to result in an increase in production. Occasionally multiple copy number can actually reduce the level of expression (Thrill et al, 1990).

As in *S.cerevisiae* 5' and 3' flanking UTRs are important for optimal expression. In the *P. pastoris* system utilizing AOX1 flanking regions it is highly desirable that the 5' UTR of the heterologous gene is identical to or adheres as close as possible to that of the natural AOX1 mRNA leader sequence. Sreekrishna et al (1990) found that expression levels of HSA were increased 50 fold on adjusting the 5' UTR so that it was identical in sequence and length to the natural AOX1 mRNA. Furthermore, it has been shown that genes rich in AT content are sometimes not efficiently transcribed in *P. pastoris* due to the presence of sequences that act as a transcription block. One such sequence was identified in the HIV-gp120 gene which when altered to decrease the AT content enabled transcription and subsequent expression of the protein to be achieved (Scorer et al, 1993). Synthetic genes using *P. pastoris* preferred codons can be designed for optimal expression (Brankamp et al, 1995).

For optimal secretion from *P. pastoris* using the  $\alpha$ -factor signal peptide it has been found to be useful to retain 'Glu-Ala spacers' adjacent to the KEX2 protease cleavage site in the sequence Lys-Arg-Glu-Ala-Glu-Ala. KEX2 cleavage occurs between the arginine and glutamate residues and the Glu-Ala spacers are subsequently removed by a diaminopeptidase similar to the *STE13* gene product in *S. cerevisiae*. It is thought that the spacers help to reduce steric effects caused by the fused heterologous protein which can interfere with efficient cleavage of the secretion signal (Thrill et al, 1990).

As in *S. cerevisiae* protein stability can be improved by using protease deficient strains of *P. pastoris* such as



SMD1163 (*pep4*, *prb1*). Such strains have proved their importance in the expression of ghilanten in *P. pastoris* for example (Brankamp et al, 1995), and in the efficient expression of insulin-like growth factor-1, production of which in *P. pastoris*, has been patented (Brierley et al, 1994).

The fermentation medium can also be manipulated to improve production. The pH of the medium in particular affects the stability of secreted products. The secretion of human serum albumin was enhanced by raising the pH of the medium from 5.2 to 6.0 (Sreekrishna et al, 1990). Addition of casamino acids to the medium has also been shown to contribute to increased product stability. Clare et al. (1991b) found that optimal recovery of mouse epidermal growth factor occurred at a pH of 3.0 with casamino acids added to the medium.

A minor problem with the AOX1 replacement system in *P. pastoris* is that AOX1 deleted transformants need to be grown in the presence of methanol to induce the AOX1 promoter but lack of the alcohol oxidase enzyme results in poor growth when methanol is used as the sole carbon source. The solution has been to stimulate cell productivity on alternative carbon sources and then switch to methanol to induce production. This technique is generally used with inducible promoters in *S. cerevisiae* where the product may be toxic and may, of course, be desirable when using *P. pastoris* for the same reason depending on the nature of the product. Continuous production can be achieved by using mixed media with more than one carbon source, methanol and glycerol for example, but whilst this successfully increases biomass, expression levels are somewhat restricted due to partial repression of the AOX1 promoter by glycerol (Sreekrishna et al, 1989). For continuous fermentation on methanol it may be preferable to use transformants retaining a copy of the

*AOX1* gene. Such transformants occur naturally during integration events using 5' and 3' *AOX1* containing vectors but may be obtained by targeted integration of the entire vector into *AOX1* or *HIS4* loci using a single restriction cut at a suitable site within the region of homology.

#### 1.5.8. A Yeast Expression System for Insect AChE

In addition to the general advantages of expression in yeast for the study of protein structure and function and the accompanying biotechnological potential of recombinant AChE production in *S. cerevisiae*, for the aims of the current project a yeast expression system also offers the powerful and time saving possibility of direct transformation with synthetic oligonucleotides. Rather than employing the conventional technique of using a single stranded *E. coli* vector and isolating recombinants containing the desired mismatch in *E. coli* for each specific alteration required, before subcloning back into a vector suitable for expression, a single step procedure of mutagenesis *in vivo* could be attempted. In this way yeast expressing AChE would be transformed solely with synthetic oligonucleotides containing mutations and subsequently screened for resistance. Moerschell *et al* (1988) have reported the successful site-directed mutagenesis *in vivo* of the iso-1-cytochrome c gene of *S. cerevisiae* in *S. cerevisiae* using direct transformation with synthetic oligonucleotides. The mechanism remains unclear but transformants are thought to arise by invasion of the homologous parental sequence by the single stranded oligonucleotide during DNA replication. The site-directed mutagenesis reported by Moerschell *et al* was accomplished with a chromosomal gene (*CYC1*) rather than a plasmid borne gene, but the behaviour of a single copy number centromeric plasmid such as pBM150 during mitosis and meiosis is similar to that of a mini chromosome and a plasmid such as pPIC9 integrates the gene of interest into the yeast



chromosome. A high copy number plasmid would present problems for direct transformation since mutants containing new recombinant plasmids also carry plasmids containing the wild type gene. In the event of successful expression from a YE<sub>p</sub> plasmid such as pG3 or p2UG it may be possible to introduce a centromere into the vector, thereby reducing the copy number to 1 - 2 copies per cell, and making the system more suitable for direct transformation. Expression from a high copy number vector would still, of course, be conducive to conventional methods of site-directed mutagenesis if required.

Difficulties were encountered in the setting up of the system, both in terms of cell wall interference in the assay of choice for AChE (Ellman et al, 1961), and in terms of subcloning problems, given the length of the gene and the limited number of restriction options within the polylinkers of the yeast expression vectors available. Necessarily then, the project mainly focused on achieving the expression of the *D. melanogaster* AChE (DAChE), the cDNA of which was already available (Hall and Spierer, 1986).

Successful expression of the DAChE gene was achieved under the constitutive *GPD* promoter using the high copy number yeast episomal plasmid pG3. Expression levels from *S. cerevisiae* protoplasts, although low, were found to be well within the range of sensitivity of the Ellman assay and inhibition of the expressed enzyme was demonstrated with both the insecticide Bendiocarb and the irreversible cholinesterase inhibitor phenylmethylsulfonyl fluoride (PMSF).

A yeast based system for insect AChE suitable for mutagenesis studies is now available in *S. cerevisiae* using the constitutive vector pG3 but a higher level of expression would be desirable. Results were indicative of

low levels of a biologically active enzyme in the correct conformation. Active AChE can be released from yeast protoplasts and used directly in microtitre plate assays for inhibitor studies. This immediately makes the system in *S. cerevisiae* more convenient than the alternative microbial system developed in *E. coli* (Fischer et al, 1993) because the *E. coli* enzyme needs to be refolded *in vitro* to achieve activity.

The expressed DACH<sub>E</sub> was found to be located in the cell membrane of *S. cerevisiae* and the active site is probably situated on the inside rather than the outside of the membrane. Further characterisation of the expressed enzyme is warranted but for the long term aims of the project two priorities remain: the first being the enhancement of DACH<sub>E</sub> expression in *S. cerevisiae* and the second being isolation of a susceptible mosquito AChE cDNA from *Cx. molestus*. RT-PCR was employed to generate a homologous probe that could in the future be used for the screening of a cDNA library from *Cx. molestus* as a first step to the isolation of a susceptible mosquito cDNA. Attempts to subclone the DACH<sub>E</sub> gene into the vectors p2UG and pPIC9 were unfortunately not successful but alternative manipulations should be tried because levels of expression need to be significantly enhanced before large scale production becomes feasible. Although expression levels of vertebrate AChE were found to be low using pPIC9 in *P. pastoris* (Morel and Massoulie, 1997) the expression and secretion of an invertebrate AChE using the same system may differ considerably.



## CHAPTER TWO MATERIALS AND METHODS

Unless otherwise stated the cloning procedures given below are based on standard protocols to be found in laboratory manuals (Maniatis *et al*, 1982; Berger and Kimmel, 1987; Guthrie and Fink, 1991) or as given by the manufacturers of standard molecular cloning 'kits'. Except where otherwise stated general purpose reagents were purchased from Sigma or BDH. For DNA work all solutions and buffers where necessary were sterilized by autoclaving (15 psi, 15 min) or filtering, and where possible presterilized plasticware was used throughout. Plasticware or glassware that had not been presterilized was autoclaved prior to use. Disposable plastic gloves were used for all DNA procedures. RNA work required more stringent procedures and these are given in the appropriate sections. All handling of radioactive substances was performed in a separate laboratory specifically designed for this purpose and disposal carried out in accordance with guidelines set out by the Departmental Radiation Protection Officer.

### 2.1. Mosquitoes

A strain of autogenous *Culex molestus* (Elephant) originally isolated from the London Underground was maintained at room temperature in nylon mesh cages and fed on a solution of 10% w/v glucose. Eggs laid in small plastic bowls containing water were allowed to hatch and the larvae transferred to larger trays for rearing. Larvae were fed on guinea pig food pellets. Pupae were transferred back to the cages in small bowls for emergence. Batches of insects were frozen down and stored in liquid nitrogen (BOC).

### 2.2. Yeast Strains

The *Saccharomyces cerevisiae* strains EYL363-6D (*MATa ura3-*

52 *leu2*  $\Delta$ ade 2-101 *lys2-52 cyhr*) (Rhona H. Borts, Rosentiel Center, Brandeis University) and BJ2168 (*MATa leu2 trp1 ura3-52 prb1-1122 pep4-3 prc1-407 gal2*) (Yeast Genetics Stock Center: MCB/Biophysics and Cell Physiology, 102 Donner Laboratory, University of California, Berkeley, CA 94720) were grown and maintained on sterile Yeast Minimal Medium (M.M.) (0.67% w/v Bacto-yeast nitrogen base (without amino acids) (Difco), 2% w/v glucose) supplemented with appropriate amino acids (Sigma) as required. EJL363-6D: adenine (20mg/l); leucine (30mg/l); lysine (20mg/l); uracil (20mg/l). BJ2168: uracil (20mg/l); leucine (30mg/l); tryptophan (20mg/l). 2% w/v agar (Oxoid) was added for solid media and 1M sorbitol added for regeneration media. Transformed and untransformed strains were kept on agar plates at 4°C and subcultured every 4 weeks.

### 2.3. *Escherichia coli*

*E. coli* strain DH5 $\alpha$  was propagated in Luria Bertani (L. B.) Medium (1% w/v bactotryptone (Oxoid), 0.5% w/v yeast extract (Beta Lab), 1% w/v NaCl, 0.1% v/v 1N NaOH) and *E. coli* transformants selected on L. B. Medium containing 2% w/v agar and ampicillin (Sigma) (75 $\mu$ g/ml).

### 2.4. Plasmids and DNAs

*D. melanogaster* AChE cDNA (Hall and Spierer, 1986) with leader sequence deleted (first ATG is start) 3.3Kb long (Hall, pers.comm., 1991) in *EcoRI* site of Bluescript KS+ (2.9Kb) (Stratagene) to give recombinant plasmid of 6.2Kb (pKS-DACH). Details of the cDNA sequence are given in chapter four. *S. cerevisiae* shuttle vectors used in subcloning were: p2UG (6.0Kb) (Figure 2.1) and pG3 (9.1Kb) (Figure 2.2) (Schena, et al 1991) derived from the *E. coli* pUC18 plasmid and sections of yeast DNA including the 2 $\mu$ m origin of replication and *URA3* and *TRP1* genes for p2UG and pG3 respectively. pG3 is a constitutive expression vector



utilizing the *GPD* promoter and contains stop codons in all three reading frames in addition to the *S. cerevisiae* PGK transcription terminator and polyadenylation signal. p2UG is a steroid inducible vector containing three GREs fused upstream of the *CYC1* promoter. *Bam*HI and *Sac*I linkers were purchased from New England Biolabs. pBM150-DACHE, a centromeric plasmid construct containing the DACHE cDNA cut from pKS-DACHE with *Hinc* II and *Sma* I, inserted into the *Eco*RI site of pBM150 (8.26Kb) (Figure 2.3) (Johnston and Davies, 1984) using *Eco*RI polylinkers (Curran, pers.comm., 1991), and transformed into the yeast strain EJL363-6D was already available within the laboratory. The *Pichia pastoris* vector pPIC9 (8.0Kb) (Figure 2.4) (Invitrogen) is an integrative plasmid utilizing the *AOX1* promoter and containing the  $\alpha$ -factor secretion signal. Integration can be directed to either the *AOX1* locus or the *HIS4* locus of *S. cerevisiae* depending on whether the vector is cut with *Bgl*III or *Sal*I (Figure 2.4). The double stranded oligonucleotide with *Xho*I and *Pvu*I restriction sites designed for subcloning the DACHE cDNA into pPIC9 was synthesised by Genosys. Primers homologous to genomic sequences of *Cx. pipiens* for RT-PCR were also synthesised by Genosys.

## 2.5. Preparation of Competent *E. coli* Cells for Transformation

5ml of L. B. Medium (2.3) in a 15ml Falcon tube was inoculated with a single colony of *E. coli* and grown overnight with shaking at 37°C. Next morning 250ml of L. B. Medium was inoculated with the 5ml overnight culture and grown to an OD<sub>600</sub> of between 0.5 and 1.0. The cells were harvested by centrifuging at 5000 rpm for 5 min at 4°C (Beckman J2-21). The supernatant was discarded and the cells resuspended in 125ml of cold 50mM CaCl<sub>2</sub>. The cells were left on ice for 20 min and subsequently pelleted at 5000 rpm for 5 min at 4°C. The cell pellet was resuspended

Figure 2.1. The high copy number steroid inducible episomal plasmid p2UG (after Picard et al, 1990).



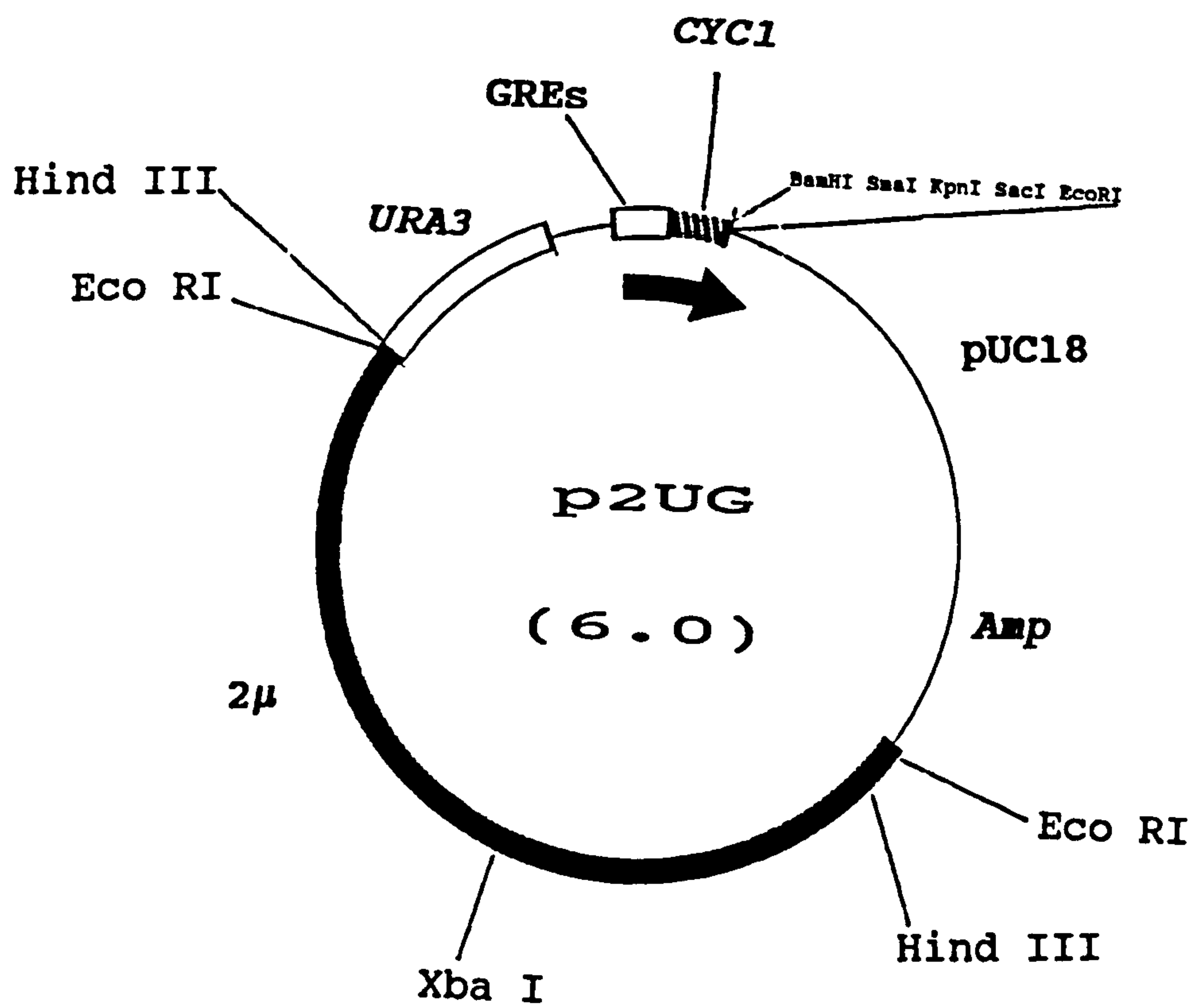


Figure 2.2. The high copy number episomal plasmid pG3 (after Schena and Yamamoto, 1988; Schena et al, 1989). Between the two *EcoRI* restriction sites in the polylinker there is a 1.7Kb 'stuffer fragment' (indicated as a dotted line).



Bam HI Sma I Kpn I Sac I Eco RI....Eco RI Sac I Kpn I Sma I Bam HI Sal I

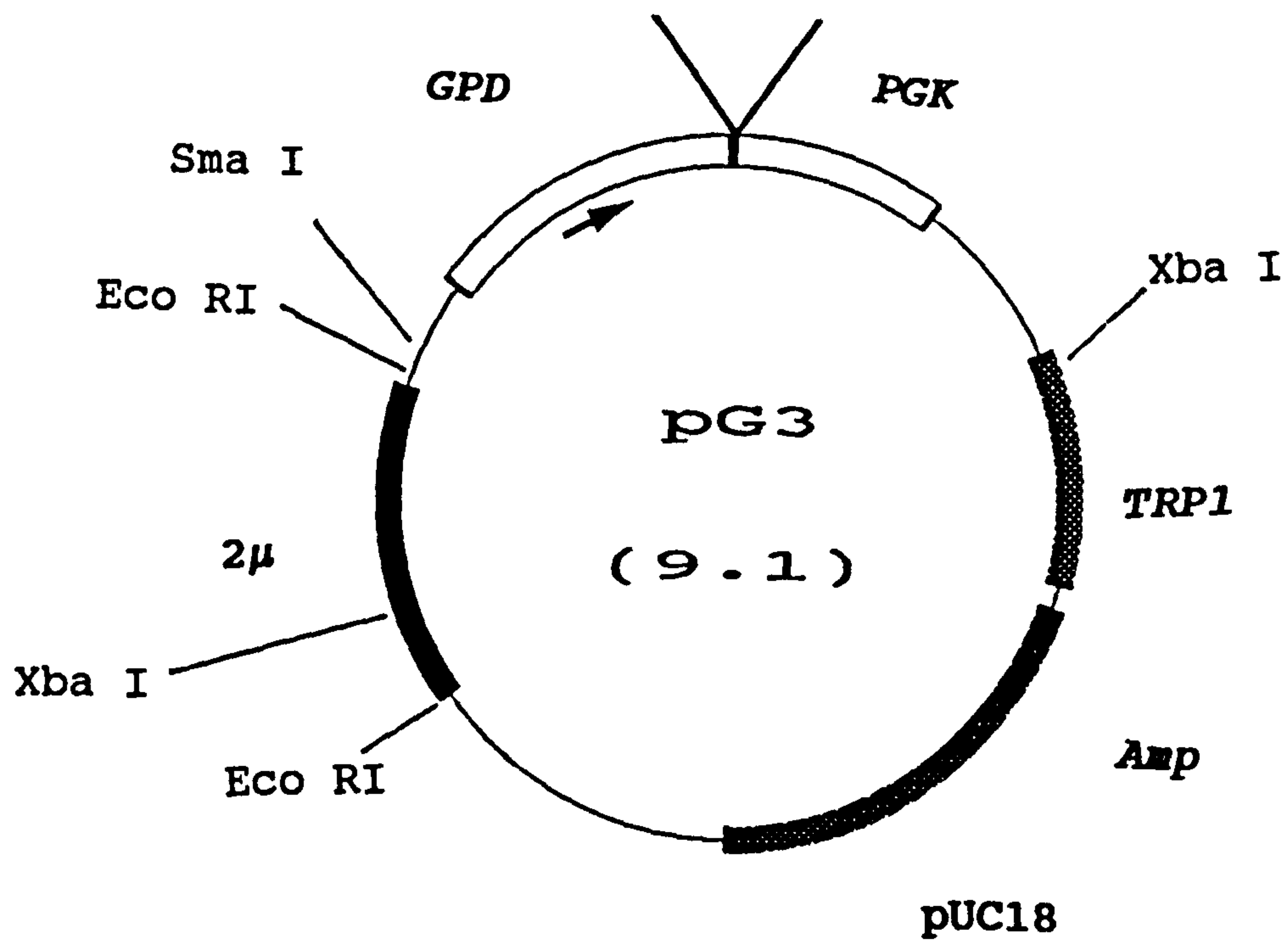


Figure 2.3. The single copy centromeric plasmid pBM150. Subcloning into the *EcoRI* restriction site places the gene of interest under the inducible *GAL10* promoter (after Johnston and Davies, 1984).



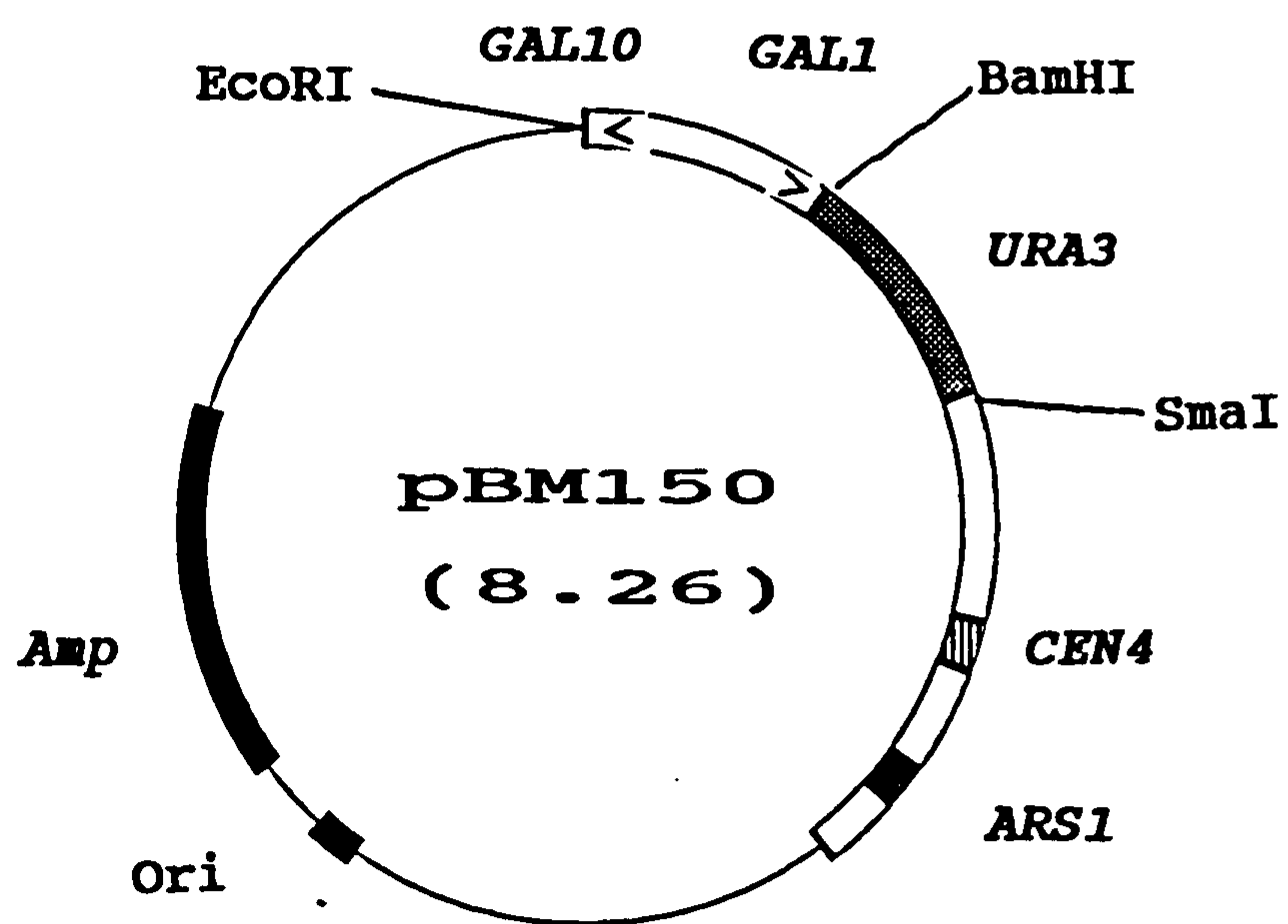
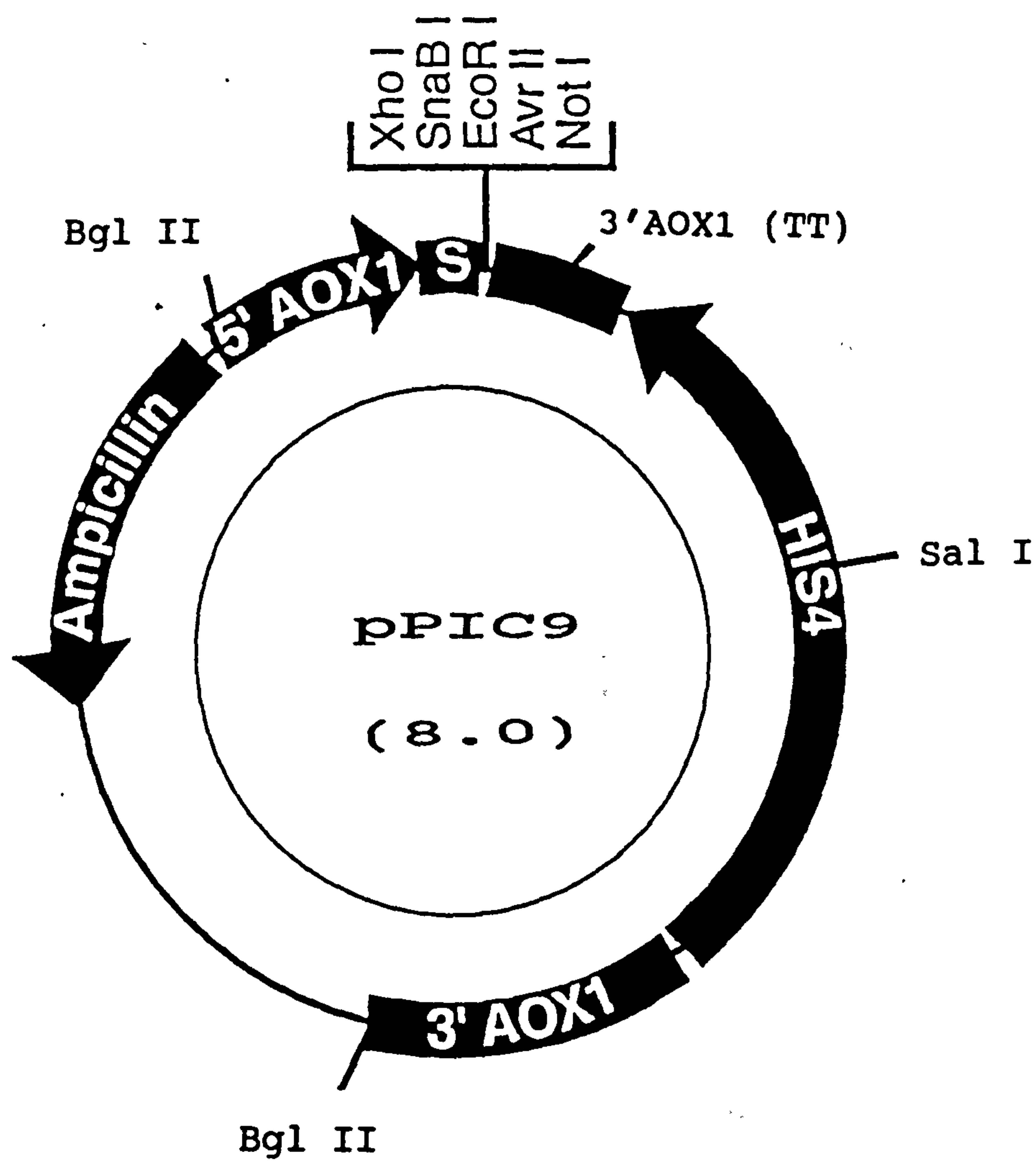


Figure 2.4. The secretion plasmid pPIC9 designed for use with the yeast *Pichia pastoris* (Invitrogen). The AOX1 promoter is induced when *Pichia pastoris* is grown on media containing methanol instead of glucose.

S =  $\alpha$ -factor secretion signal.





in 20ml cold 50mM CaCl<sub>2</sub>, and left on ice for 4 hr. 7ml of 50% glycerol was added to the cells and carefully mixed. Immediately 0.5ml aliquots were transferred to cold Eppendorph tubes and the aliquots stored at -70°C. Care was taken throughout to ensure that the cells were kept cold. Competency was assessed by transforming cells with a plasmid such as pUC19.

## 2.6. Transformation of *E. coli*

An aliquot of cells was removed from the -70°C freezer and allowed to thaw on ice. Between 100 and 500ng of DNA was used per transformation. Ligation mixtures were added directly. The DNA was added to 200μl of competent cells in a 0.5ml Eppendorph tube, mixed, and the cells left on ice for 20 min. A control tube of cells without DNA added was taken through the same procedure. Cells were heat shocked at 42.5°C for 3 min using a heating block. Cells were left for 10 min at room temperature, transferred to a 1ml Eppendorph, 1ml sterile L. B. Medium (2.3) added, and incubated at 37°C for 1 hr. Cells were pelleted in a microfuge (MSE 13000 rpm, 3 min), resuspended in 50μl of sterile L. B. Medium, and plated on L. B. Medium containing the appropriate antibiotic. Plates were incubated overnight at 37°C.

## 2.7. Transformation of *S. cerevisiae* with Expression Plasmids by Electroporation (after Becker and Guarente, 1991)

A single colony of the *S. cerevisiae* strain to be transformed was inoculated into 5ml YPD medium (1% w/v yeast extract, 2% w/v bactopectone (Oxoid), 2% w/v glucose) and grown to stationary phase at 27°C. An aliquot was taken and used to inoculate a further 500ml YPD medium and the culture grown to an OD<sub>600</sub> of 1.5 at 27°C. The culture was split into two sterile 250ml centrifuge bottles and the



cells harvested at 5000 rpm for 5 min at 4°C (Beckman J2-21). The supernatants were discarded and the cells resuspended in 250ml ice cold sterile distilled water (SDW). Cells were reharvested at 5000 rpm for 5 min at 4°C. Supernatants were discarded and the cells resuspended in 125ml ice cold SDW and then the two aliquots pooled to make a final volume of 250ml. The cell suspension was recentrifuged and the supernatant discarded. The pellet was resuspended in 20ml ice cold 1M sorbitol, transferred to a chilled 30ml Corex glass tube, and spun at 5000 rpm for 5 min at 4°C (Beckman J2-21). The supernatant was discarded and the pellet resuspended in 0.5ml ice cold 1M sorbitol. Yeast cells were kept on ice throughout the electroporation procedure.

Yeast cells were electroporated using a Biorad Gene Pulser and sterile Bio Rad 0.2cm cuvettes. A 40 $\mu$ l aliquot of yeast cell suspension was used for each transformation. To each yeast cell aliquot in a sterile Eppendorph tube was added between 100 and 500ng of expression plasmid DNA in a volume of no more than 5 $\mu$ l. After mixing gently the tube was incubated on ice for 5 min. The yeast and DNA were transferred to a cold electroporation cuvette, tapped to the bottom, and pulsed at 1.5kV, 25 $\mu$ F, 200 $\Omega$ . Immediately 1ml of ice cold 1M sorbitol was added to the cuvette and the contents gently mixed using a Gilson pipette before transferring to a sterile Eppendorph tube. It was found to be vital for good transformation efficiencies that the cells were plated immediately following electroporation. If a number of replicas and controls were to be transformed then individual aliquots were pulsed and plated rather than electroporating all and then plating. Cells were plated on sterile selective M.M.(2.2) containing 1M sorbitol and incubated at 27°C until colonies appeared.

## 2.8. Isolation of Plasmids from *E. coli*

### 2.8.1. Large Scale Alkaline Lysis Method

This procedure is suitable for the preparation of large quantities of plasmid and is detailed below for an overnight culture of 50 ml. It was scaled up as required.

A 50ml culture of plasmid bearing bacteria was grown overnight at 37°C in L. B. Medium (2.3) with the appropriate antibiotic. The bacteria were harvested (15 min, 5000 rpm, 4°C) using a Beckman J2-21 centrifuge. The pellet was resuspended in 2ml GTE solution (50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA), 3ml 10mg/ml lysozyme (Sigma) in GTE added, and the suspension incubated in ice for 10 min. 10ml 1% w/v SDS, 0.2M NaOH was added, the suspension mixed gently and left on ice for not more than 10 min. 7.5ml 3M KOAc pH 4.8 was added to neutralize the lysed bacterial suspension and then left on ice for at least 10 min. The bacterial DNA-SDS-protein complex was then spun down by centrifugation at 8000 rpm for 10 min at 4°C. The supernatant was decanted into a fresh tube and respun at the same speed for a further 10 min. The supernatant was transferred to a fresh tube through sterile gauze and 10ml isopropylalcohol (IPA) added. Plasmid DNA, bacterial RNA, and any remaining protein was spun down at 8000 rpm for 5 min at 4°C. The pellet was resuspended in 2ml SDW, 2ml 5M LiCl, 50mM Tris pH 7.6 added, and the suspension left on ice for 30 min. The precipitated RNA was spun down at 8000 rpm for 5 min at 4°C. The supernatant was transferred to a fresh tube, 9ml ice cold absolute EtOH added, and the plasmid DNA precipitated for 1 hr at -20°C. It was recovered by centrifugation at 8000 rpm for 15 min. The supernatant was discarded and the pellet dried under vacuum for 10 min. The pellet was then resuspended in 250μl TE (10mM Tris-HCl pH 7.6, 1mM EDTA) containing 20μg/ml RNase A, transferred to an Eppendorph tube, and incubated at 37°C



for 30 min. Plasmid DNA was purified from remaining protein by adding 500 $\mu$ l phenol (equilibrated as described in Maniatis et al, 1982): chloroform: isoamylalcohol (50:49:1), vortexing, and spinning down in a microfuge (MSE) at 13000 rpm for 5 min. The upper aqueous phase was transferred to a fresh Eppendorph tube and the plasmid DNA precipitated at -20°C for 30 min with 125 $\mu$ l 7.5M NH<sub>4</sub>OAc pH 5 and 500 $\mu$ l ice cold absolute EtOH. Plasmid DNA was recovered by microfuging at 13000 rpm for 10 min, washed with 70% v/v EtOH, washed with absolute EtOH, dried under vacuum for 10 min and resuspended in 205 $\mu$ l TE.

#### 2.8.2. Small Scale Alkaline Lysis Method ('Minipreps')

Individual bacterial colonies were picked with sterile cocktail sticks and each stick dropped directly into 15ml Falcon tubes containing 5ml L. B. Medium and the appropriate antibiotic. The cultures were grown overnight at 37°C. Bacteria were harvested at 3500 rpm for 5 min using a benchtop centrifuge (MSE Centaur 2). Cocktail sticks and 4ml of each supernatant were discarded and the pellets resuspended in the remaining 1 ml of supernatant. These 1ml suspensions were transferred to Eppendorph tubes and the bacteria reharvested by microfugation at 13000 rpm for 3 min. Supernatants were discarded and each pellet resuspended in 100 $\mu$ l GTE (2.8.1) containing 2mg/ml lysozyme and the suspensions incubated in ice for 10 min. 200 $\mu$ l 1% w/v SDS, 0.2N NaOH was added, the suspensions mixed gently and left on ice for 10 min before the addition of 150 $\mu$ l 3M NaOAc pH 5.3. The tubes were incubated at -20°C for 10 min and then microfuged at 13000 rpm for 15 min. Supernatants were transferred to fresh Eppendorph tubes and respun for an additional 10 min. Supernatants were transferred to fresh Eppendorph tubes and extracted with an equal volume of phenol: chloroform: isoamylalcohol (50:49:1). Aqueous phases were transferred to fresh tubes and the plasmid DNA precipitated with 2 vols of ice cold EtOH and 0.3M NaOAc pH

5.3. Plasmid DNA was recovered immediately by microfuging at 13000 rpm for 15 min. Pellets were washed in 70% v/v EtOH, washed in absolute EtOH, dried under vacuum for 10 min and resuspended in 50 $\mu$ l of TE (2.8.1) or SDW containing RNase A at a concentration of 10 $\mu$ g/ml.

## 2.9. Extraction of Genomic DNA from *S. cerevisiae*

A 100ml culture of yeast was grown overnight at 27°C on M.M. (2.2) with appropriate amino acid supplements to an OD<sub>600</sub> of 0.1. The cells were harvested by centrifugation (3500 rpm, 5 min, MSE Centaur 2), the pellet resuspended in 1ml of SDW, and transferred to an Eppendorph tube. The cells were repelleted by spinning in a microfuge at 13000 rpm for 1 min and the cells protoplasted by resuspension in 150 $\mu$ l SZB (1M sorbitol, 100mM sodium citrate, 60mM EDTA, 1.5mg/ml Zymolyase 20,000 (Seikagaku Corporation), 100mM  $\beta$ -mecaptoethanol) and incubation at 37°C for 45 min with occasional shaking. After 20 min cells were checked for protoplasting by one of two methods: either a small amount of suspension was added to a drop of 5% v/v Triton X-100 on a microscope slide and examined for lysed cells or 10 $\mu$ l taken, added to 1ml of SDW, and tested for 'clearing' using a colorimeter. Zymolyase treatment was considered complete when greater than 80% of the cells were protoplasted. 150 $\mu$ l SDS-TE solution (2% w/v SDS, 0.1M Tris-HCl pH 8.0, 10mM EDTA) was added to the protoplasts prior to vortexing and incubation at 60 - 65°C for 10 min. 150 $\mu$ l 5M KOAc pH 5 was added, the suspension briefly vortexed, and then incubated in ice for 45 min. The suspension was microfuged for 15 min at 4°C, 300 $\mu$ l of supernatant retained, and the pellet discarded. To the supernatant were added 200 $\mu$ l 5M NH<sub>4</sub>OAc and 1ml prechilled IPA prior to incubation at -20°C for 10 min then microfuging (13000 rpm) for 5 min. The supernatant was discarded and the pellet rinsed with 80% v/v EtOH before drying under vacuum for 5 min. The pellet was redissolved in 200 $\mu$ l SDW, 2 $\mu$ l 10mg/ml RNase A added,



and incubated at 37°C for 10 min. 4 $\mu$ l 5M NaCl, 2 $\mu$ l 20mg/ml pronase were added and incubation carried out at 37°C for a further 10 min. Extraction with 200 $\mu$ l phenol: chloroform: isoamylalcohol (50:49:1) was carried out and the aqueous phase transferred to a fresh Eppendorph tube. The solution of DNA was made 0.3M with respect to NaOAC pH 5.3 and precipitated with 2 vol of EtOH. DNA was recovered by microfuging at 13000 rpm for 15 min, washed in 70% v/v EtOH, washed in absolute EtOH, dried under vacuum for 10 min, and resuspended in 50 $\mu$ l TE (2.8.1).

## 2.10. Quantification of DNA

The concentration of DNA in any given preparation was measured by absorbance spectrophotometry. A 5 $\mu$ l aliquot was taken, diluted in 1ml of TE (2.8.1), decanted into a quartz cuvette, and the absorbance read on a UV spectrophotometer (Pye Unicam PU 8600). The absorbance of a DNA solution was determined at 260 and 280nm using an absorbance coefficient of 50 $\mu$ g/ml = 1  $A_{260}$  unit. The method is subject to interference by RNA and protein and an assessment of the purity of a sample can be gauged from the  $A_{260}/A_{280}$  ratio.

## 2.11. Restriction Enzyme Digests of DNA

The following procedure was used for a typical 20 $\mu$ l reaction containing 0.1 - 1 $\mu$ g of DNA and was scaled up appropriately for larger amounts of DNA or for the digestion of linkers during subcloning. The DNA solution was placed in a sterile Eppendorph tube and mixed with sufficient SDW to give a volume of 16 - 17 $\mu$ l. 2 $\mu$ l of the appropriate restriction enzyme digestion buffer was added and mixed by tapping the tube. 1 $\mu$ l (10 - 20 Units) of restriction enzyme was added and mixed by tapping the tube. The reaction was incubated at the recommended temperature (usually 37°C) for 1 hr. The reaction was stopped by adding

5 - 10 $\mu$ l 2X Endo.R.Stop solution (50mM EDTA, 0.2% w/v SDS, 50% v/v glycerol, 0.05% w/v bromophenol blue). Digests were then either analyzed directly by agarose gel electrophoresis or purified. Purification was either by phenol:chloroform extraction followed by EtOH precipitation or by means of 'Prep-a-Gene' (Biorad) or 'Spinbind' (FMC BioProducts) purification kits in accordance with the manufacturer's instructions.

## 2.12. Agarose Gel Electrophoresis

TAE buffer (40mM Tris-Acetate, 1mM EDTA) was used for the separation of DNA bands in agarose. A 50X stock solution was made and diluted with SDW to 1X immediately prior to use for the preparation of gels and running buffer. The required percentage (0.8% w/v unless otherwise stated) of agarose was prepared in 1X TAE of a volume appropriate to the gel to be cast. A microwave was used to melt the agarose which was then allowed to cool to about 60°C before casting. The gel was allowed to set. 1X TAE buffer was prepared and the electrophoresis tank (Biorad or Pharmacia) filled to a level not more than 1-2mm above the surface of the gel. The comb was then removed. DNA samples were loaded with an appropriate volume of 2X Endo.R.Stop solution (2.11) and electrophoresed at 20 mA. Following electrophoresis the gel was removed and stained with ethidium bromide (0.1 $\mu$ g/ml in 1X TAE) for 30 min. DNA bands were visualized by placing the gel on a UV transilluminator and inducing fluorescence of ethidium bromide molecules that had intercalated into the DNA during staining. The fluorescence was recorded using a computer camera (Mitsubishi).

## 2.13. Purification of DNA Bands from Agarose Gels

For the purposes of subcloning particular fragments of DNA separated by restriction digest and agarose gel



electrophoresis needed to be purified prior to ligation of linkers or ligation into different cloning vectors. This was accomplished by cutting out the DNA band of interest from the gel using a razor blade. A hand held UV light source was used to visualize the DNA to prevent 'nicking'. The gel slice containing the fragment of interest was then transferred to a sterile Eppendorph and heated to 65°C to melt the agarose. The DNA was purified from the agarose by one of two purification methods ('Prep-a-Gene' or 'Spinbind') both of which use a glass matrix to bind the DNA. Purification was carried out in accordance with the manufacturer's instructions.

#### 2.14. Methylation of DNA for the Purposes of Subcloning

Site-specific methylases such as *Bam*HI methylase were purchased from New England Biolabs and the AChE gene methylated within Bluescript KS+ as follows:

Methylase Buffer (10X)	10 $\mu$ l
SAM* (to give final conc. 80 $\mu$ M)	2.5 $\mu$ l
pKS-DACHE (30 $\mu$ g)	30 $\mu$ l
Methylase	15 $\mu$ l
SDW	42.5 $\mu$ l
Final Volume	100 $\mu$ l

\* SAM - S-adenosylmethionine

2 Units of methylase were used per  $\mu$ g of DNA and the reaction incubated at 37°C for 1 hr. Following incubation the methylated DNA was phenol extracted as follows: 500 $\mu$ l of 10mM tris-HCl (pH 7.5) was added to the reaction in a sterile Eppendorph tube followed by 500 $\mu$ l phenol:chloroform:isoamylalcohol (50:49:1). After brief vortexing and microfugation the aqueous layer was transferred to a fresh sterile Eppendorph tube, 50 $\mu$ l of 2M NaCl added, and

the methylated DNA precipitated with 1 vol IPA for 1 hr at -20°C. The DNA was pelleted in a microfuge (13000 rpm, 15 min), rinsed gently with 100% IPA, and the pellet air dried at room temperature. Once dry the DNA was resuspended in 50µl SDW. Successful methylation was assessed by a restriction enzyme digest of an aliquot of methylated DNA which was subsequently analyzed by agarose gel electrophoresis.

## 2.15. Ligations

### 2.15.1. Ligation of Linkers to *D. melanogaster* AChE Gene

Chemically phosphorylated linkers were used throughout. The ratio of ends for the linker ligation reaction was assessed and linkers always added in excess (>2 orders of magnitude). A typical reaction was set up as follows:

Blunt Ended AChE Fragment (2.5µg)	10.0 µl
Phosphorylated Linkers (1µg)	1.0 µl
10X T4 DNA Ligase Buffer (Containing 10mM ATP)	2.5 µl
SDW	10.5 µl
T4 DNA Ligase (400 Units - New England Biolabs)	1.0 µl
Final Volume	25.0 µl

The reaction was incubated at 4°C for 48 hr. Successful ligation was checked by taking a 1µl aliquot and running on a 2% w/v TAE agarose gel (2.12). A 'smear' of ligated linker fragments should be seen after staining with ethidium bromide (0.1µg/ml in 1X TAE).

Following incubation the ligase was inactivated by heating to 65°C for 15 min. The ligation mixture was cooled on ice and 'sticky ends' generated by digestion with the appropriate enzyme as follows. The 25µl volume was made up to a final volume of 125µl with SDW and 12.5µl of the



appropriate 10X enzyme restriction buffer. 50 Units of restriction enzyme were added and the digest incubated at 37°C for 4 hr. An additional 10 Units of enzyme were added and the digest incubated for a further 1 hr. At the end of the incubation linker fragments were removed by Spinbind purification according to the manufacturers instructions.

#### 2.15.2. Recovery of Oligonucleotides by Ultracentrifugation

The strategy adopted for subcloning into plasmid pPIC9 (4.2) required restriction of a specially designed oligonucleotide prior to ligation with the DChE cDNA. However, the oligonucleotide was too small to bind successfully to the glass matrices employed in commercial DNA purification kits such as Spinbind or Prep-a-Gene. Purification therefore was achieved using phenol:chloroform followed by EtOH precipitation and recovery by ultracentrifugation.

Following restriction of the oligonucleotide (4X 3µg with 25 units of PvuI for 4 hr at 37°C, then with an additional 5 units of PvuI for 1 hr) and purification with an equal volume of phenol:chloroform:isoamylalcohol (50:49:1) the aqueous phase was transferred to a fresh Eppendorph tube, made 0.3M with respect to NaOAc pH 5.3 and 2 vol of absolute EtOH added. Precipitation was overnight at -20°C. The sealed Eppendorph tube containing ethanolic solution and restricted oligonucleotide was placed in a Beckman ultracentrifuge tube that had been three quarters filled with SDW. Centrifugation was then carried out at 27000 rpm for 2 hr at 4°C (Beckman L5-50, SW28 rotor). Following centrifugation the Eppendorph tube was carefully removed and the supernatant aspirated off. The pellet was then washed with 70% (v/v) EtOH and recovered by microfuging at 13000 rpm for 15 min at 4°C.

### 2.15.3 Ligation of AChE Gene Fragment to Vectors Carrying Compatible Termini

Compatible termini were generated in expression vectors by cutting in the polylinker with the appropriate restriction enzyme followed by isolation of the vector by means of gel purification (2.13). Ligations were set up in varying ratios and quantities of vector and gene fragments, but typical reactions involved the ligation of approximately 300ng of vector to 100ng gene fragment (3:1 ratio) or 200ng of vector to 100ng gene fragment (2:1) or 200ng of vector to 200ng gene fragment (1:1). The ratio selected depended on whether a ligation involving a phosphatased vector (see 2.16) was used or an unphosphatased vector. Typically a 3:1 ratio was used with the former and a 1:1 with the latter. The reactions were set up on ice as follows:

Gene Fragment in SDW (100ng)	8 $\mu$ l
Vector in SDW (200ng)	2 $\mu$ l
10X T4 DNA Ligase Buffer (Containing 10mM ATP)	2 $\mu$ l
SDW	7 $\mu$ l
T4 DNA Ligase (4-40 Units New England Biolabs)	1 $\mu$ l
Final Volume	20 $\mu$ l

Prior to the addition of ligase the reaction mix was heated to 65°C for 2 min and then immediately cooled on ice. Ligase was added and the ligation incubated at 4°C for 48 hr. Following incubation ligations were used directly to transform *E. coli* (2.6).

### 2.16. Phosphatasing of Cloning Vectors Using Alkaline Phosphatase

Calf intestinal alkaline phosphatase (CIAP) (New England Biolabs) was used. The reaction was set up as follows:



10X CIAP Buffer	5 $\mu$ l
Vector DNA cut with eg. <i>Sac</i> I and gel purified (4 $\mu$ g)	20 $\mu$ l
SDW	23 $\mu$ l
CIAP (Units*)	2 $\mu$ l
Final Volume	50 $\mu$ l

\* The number of Units of CIAP to be used was calculated on the basis that 1.0 Unit is required per pmol ends for DNA with 3' overhangs and 0.1 Unit is required per pmol ends for DNA with 5' overhangs (New England Biolabs). 1 $\mu$ g of linear pG3 (7.4 Kb) = 0.21pmol DNA = 0.42 pmol ends: 4 $\mu$ g cut with *Sac*I (3' overhang): 1.68 pmol ends = 1.68 Units. 1 $\mu$ g of linear p2UG (6.0 Kb) = 0.25 pmol DNA = 0.5 pmol ends: 4 $\mu$ g cut with *Bam*HI (5' overhang): 2 pmol ends = 0.2 Units.

Half the number of Units of CIAP required were added and the reaction incubated at 37°C for 30 min. The remaining half of the number of Units of CIAP required were added and the reaction incubated for another 30 min. Following incubation the CIAP was inactivated by adding EDTA to 5mM and heating to 65°C for 1 hr. The phosphatased vector was purified by extraction with an equal volume of phenol: chloroform:isoamylalcohol (50:49:1) followed by EtOH precipitation as detailed in 2.8.2.

## 2.17. Blue/White Colour Selection Using pUC19

The AChE gene fragment was ligated into pUC19 (2.15.3), transformed into *E. coli* (2.6.), and plated on X-gal media. X-gal plates were prepared as follows:

Top Layer (each plate)

7ml 2% w/v Agar in L. B. Medium (2.2)

100 $\mu$ l 10mM IPTG\* (Sigma)

50 $\mu$ l 2% w/v X-gal\*\* (Sigma)

50 $\mu$ g/ml Ampicillin\*\*\* (Sigma)

#### Bottom Layer

L. B. Medium with 2% w/v Agar and 75 $\mu$ g/ml Ampicillin

\* isopropylthio- $\beta$ -D-galactoside in SDW sterilized by filtration (0.22 $\mu$ m) and stored at -20°C.

\*\* 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside 20mg/ml in dimethylformamide stored in a dark bottle at -20°C.

\*\*\* Ampicillin stock solution 100 mg/ml in SDW stored at -20°C.

#### 2.18. Southern Blotting (after Southern, 1979)

DNA fragments that had been separated according to size by agarose gel electrophoresis were denatured, neutralized, transferred to a Hybond-N (Amersham) nylon filter by Southern blotting and immobilized by baking. The blotting method preserves the relative positions of the DNA fragments and provides a suitable matrix for subsequent probing procedures. Following agarose electrophoresis and staining with ethidium bromide the gel was soaked in 200ml of 1.5M NaCl, 0.5M NaOH (denaturing solution) for 15 min. This step was then repeated with fresh denaturing solution. The denaturing solution was then discarded and replaced with 200ml 1.5M NaCl, 0.5M Tris-HCl pH 7.2, 0.001M EDTA (neutralizing solution) and the gel was soaked for 15 min. This step was then repeated. The neutralizing solution was then discarded. A capillary blot was set up as follows:

400ml of 20X SSC (3M NaCl, 0.3M Na<sub>2</sub>citrate) was poured into a dish and a glass plate positioned to make a bridge. A



piece of 3MM Whatman paper was cut to fit neatly over the glass plate so that each end was immersed in 20X SSC. The filter paper was saturated with 20X SSC. Any air bubbles between the plate and the filter paper were removed using a glass rod. The gel was inverted and placed on the saturated filter paper taking care to remove any air bubbles between the paper and the gel. A piece of Hybond-N nylon membrane was cut to the exact size of the gel and the right hand corner of the membrane snipped off in order to orientate it. The membrane was then placed carefully onto the gel using forceps. 3 sheets of 3MM Whatman paper were cut to size, wetted with 20X SSC and placed on top of the nylon membrane. A stack of paper towels (10cm high) was placed on top of the sheets of filter paper and held in place with a glass plate. A weight of between 500g and 1Kg was placed on the glass plate. 'Short circuiting' of transfer buffer between the filter paper under the gel and the paper towels was prevented by sealing the system around the gel with cling film. Transfer of DNA was allowed to proceed for 12 - 24 hr. The towels and filter papers were removed and the position of the gel slots marked on the Hybond-N with a pencil. Using forceps the membrane was transferred to a clean piece of 3MM paper and allowed to air dry. Once dry the membrane was baked between 2 sheets of 3MM paper for 2 hr at 80°C in order to fix the DNA.

## 2.19. Dot Blotting of DNA

This was used primarily as a quick method for checking the fidelity of radio labelled probes. DNA samples were heated to 95°C and chilled on ice. 1 volume of 20X SSC (2.18) was added. Samples were spotted in 2 $\mu$ l aliquots onto a small piece of Hybond-N prewetted with 10X SSC. The membrane was allowed to dry between each aliquot. The membrane was immersed in denaturing solution for 5 min and then transferred to a filter paper wad (3MM Whatman) soaked in neutralizing solution for 1 min. The membrane was allowed

to air dry and the DNA fixed by baking at 80°C for 2 hr.

## 2.20. Colony Blotting

Circular membranes of Hybond-N or Hybond N+ (Amersham) specifically designed for this purpose were used. The membrane was carefully placed onto the agar surface and a sterile needle used to make marks of orientation on the membrane and agar. The membrane was carefully removed after 1 min and placed colony side up on a pad of 3MM Whatman paper soaked in denaturing solution (2.18) for 7 min. The membrane was transferred to a pad of 3MM paper soaked in neutralizing solution (2.18) for 5 min. The membrane was then washed in 2X SSC (2.18) to remove any bacterial debris, transferred to dry 3MM paper and allowed to air dry colony side up. Once dry the membrane was oven baked at 80°C for 2 hr. If replicas were required membranes were removed from the agar and incubated at 37°C until fresh colonies were obtained. Replica colonies could be blotted as described.

## 2.21. Hybridization of DNA with Radiolabelled DNA Probes (after Wahl *et al*, 1979)

### 2.21.1. Prehybridization

Membranes were prehybridized in a plastic bag at 42°C with gentle shaking for a minimum of 1 hr in the following hybridization solution: 5X Denhardt's (0.1% w/v bovine serum albumin (BSA), 0.1% w/v Ficoll, 0.1% w/v polyvinylpyrrolidone (PVP)), 5X SSC, 50mM sodium phosphate pH 6.5, 0.1% w/v SDS, 50% v/v deionized formamide and 250µg/ml non-homologous DNA (salmon sperm DNA prepared according to the method of Maniatis *et al*, 1982). Membranes were placed in a bag (no more than 2 membranes (back to back) per bag) and hybridization solution without non-homologous DNA added at a volume of 4ml/100cm<sup>2</sup> membrane.



Non-homologous DNA was denatured at 95-100°C in a boiling water bath for 10 min and then added to the bag. Care was taken to ensure that all air bubbles were removed and the bag was then heat sealed.

### 2.21.2. Preparation of Radiolabelled DNA Probes

Radiolabelled  $^{32}\text{P}$  DNA probes were prepared using a random priming kit (Amersham). 25ng of DNA in a volume of 1-10 $\mu\text{l}$  of SDW or TE (2.8.1) was labelled with 50 $\mu\text{Ci}$  of [ $\alpha$ - $^{32}\text{P}$ ] dCTP by priming with random hexanucleotides in accordance with the manufacturer's instructions. The incorporation of  $^{32}\text{P}$  labelled dCTP (3000 Ci/mmol) in the presence of Klenow fragment DNA polymerase along with the three other unlabelled dNTPs was allowed to proceed overnight at room temperature. Then the probe was spun through a 1ml column of Sephadex-G50 to reduce unincorporated radiolabelled nucleotides and check that the DNA had been labelled. The Sephadex spun column was prepared in a sterile 1ml syringe by plugging the narrow end with sterile glass wool up to the 0.1ml mark. The syringe was placed into a 13ml polycarbonate tube and using a plastic Pasteur pipette TE saturated Sephadex G-50 added to the syringe until full. The column was spun at 3500 rpm for 5 min (MSE Centaur 2). More Sephadex was added up to the 1ml mark of the syringe. The column was respun to pack the Sephadex tightly in the syringe. The radiolabelled probe was added to the top of the spun column and centrifuged through the Sephadex at 3000 rpm for 5 min. Following centrifugation the labelled probe was transferred to a clean Eppendorph tube. The probe was denatured by boiling for 3 min and snap cooled on ice.

### 2.21.3. Hybridization

The nylon membrane to be probed was transferred to a fresh

plastic bag and fresh hybridization solution (2.21.1) (4ml/100cm<sup>2</sup> membrane) together with denatured non-homologous DNA (250µg/ml) added. The fluid was dispersed over the entire membrane and any air bubbles removed. The bag was heat sealed. A corner of the hybridization bag was snipped off and the denatured radiolabelled probe (50µl) added. Any remaining air bubbles were removed and the corner of the bag heat sealed. Care was taken to ensure there were no leaks from the bag. The probe was dispersed throughout the bag, using a Geiger counter to check that counts were uniform across the entire surface of the membrane. The bag was transferred to a 42°C water bath and hybridization allowed to proceed overnight.

#### 2.21.4. Washing of Hybridized Membranes

Following hybridization a corner of the bag was snipped off and the solution containing the radiolabelled probe drained into a sterile universal cylinder. The probe could then be stored at -20°C and re-used. The bag was cut open using scissors and the radioactive membrane carefully removed with plastic forceps. The membrane was immersed in a tray containing 200ml of wash solution 1 (2X SSC, 0.1% w/v SDS) and left at room temperature for 5 min. The wash solution was carefully decanted and the wash repeated at room temperature for a further 5 min. The membrane was washed in 200ml wash solution 2 (0.1% SSC, 0.1% w/v SDS) at 42°C for 15 min. This wash was repeated. If necessary a higher stringency wash was performed using wash solution 2 at 50-65°C.

#### 2.21.5. Autoradiography

After the second wash at 42°C the wash solution was decanted and the damp membrane transferred to a plastic bag. The bag was heat sealed and, with the DNA side of the membrane uppermost, placed in an autoradiograph cassette. Under



safe light illumination a piece of film (Fugi RX 100) of the correct size was placed on top of the membrane, an intensifying screen placed on top of this, the cassette closed and left at -70°C for a time period determined from a Geiger count assessment of the membrane. Autoradiographs were developed as follows: the cassette was allowed to warm to room temperature, opened under safe light illumination and the film removed. The film was immersed in developer for 5 min, transferred to fixative for 2 min, and washed in water for 1 min. The film was hung up to air dry.

#### **2.21.6. Removal of Radiolabelled Probe from a Nylon Membrane**

The membrane was incubated in 200ml 0.4M NaOH at 45°C for 30 min and then transferred to 200ml 0.1X SSC, 0.1% w/v SDS, 0.2M Tris-HCl pH 7.5 and incubated for a further 15 min at the same temperature. The membrane was exposed to film at -70°C overnight to check that the probe had been successfully removed.

### **2.22. Assays for the Expression of DAcE in *S. cerevisiae***

#### **2.22.1. Growth Conditions**

150ml cultures of yeast were grown in 500ml flasks at 27°C in M.M (2.2) to mid-exponential phase (OD<sub>600</sub> of 0.1). The medium was supplemented with the appropriate amino acids with the nutritional marker omitted in the case of the transformants. Each culture was split into three 50ml samples which were processed independently. Transformants containing the constitutive vector pG3 were processed directly. Transformants containing the inducible galactose vector pBM150 were harvested, washed with galactose medium (2% w/v galactose in place of 2% w/v glucose in M.M), and induced for 2 hr in 150 ml galactose M.M prior to splitting and processing.

### 2.22.2. Enzyme Extraction using Glass Beads

Cells were harvested by centrifugation at 3000 rpm for 5 min (MSE Centaur 2). The pellets were washed in 10 ml SDW and cells reharvested by centrifugation (3000 rpm, 5 min). The cells were resuspended in 1ml SDW, transferred to Eppendorph tubes, spun at 13000 rpm in a microfuge (MSE) for 1 min, and the pellets resuspended in 1 vol of phosphate buffer pH 7.0 (100 or 67mM for Ellman and Sabine assays respectively). Cells were homogenized using 1 vol acid washed glass beads (50 mesh Sigma) on a Janke and Kunkel vibrax machine (IKA-VIBRAX VXR) for 45 min at 4°C. Cells were vortexed for six 30 sec bursts, each sample chilled on ice for 30 sec between each burst.

### 2.22.3. Enzyme Extraction from Protoplasts

Cells were harvested by centrifugation at 3000 rpm for 5 min (MSE Centaur 2). The pellets were resuspended in 1 ml of 1M sorbitol, transferred to Eppendorph tubes, microfuged at 13000 rpm for 1 min, and the pellets resuspended in 150 $\mu$ l SZB (2.9), then incubated at 37°C for 30 min. Protoplasting was confirmed by diluting 10 $\mu$ l into 200 $\mu$ l of SDW in a glass test tube and observing clearing of the initially cloudy suspension. Protoplasts were spun down at 13000 rpm in a microfuge for 1 min and washed by resuspending the pellet in 1ml 1M sorbitol and respinning.

2.22.4. Ellman Assay (Ellman et al 1961) adapted for Microtitre Plate Reader (after French-Constant and Bonning, 1989).

The assay of choice for AChE is the one developed by Ellman et al (1961) which makes use of a thiol derivative of acetylcholine in the following reaction:



## AChE

Acetylcholine -----> Thiocholine + Acetate

Thiocholine + Dithiobisnitrobenzoate -----> Yellow Colour

The yellow anion is 5-thio-2-nitrobenzoic acid and its formation can be measured by absorbance between 400 and 420nm on a spectrophotometer.

Kinetic assays on DACH released from burst protoplasts were measured using a microtitre plate reader (Anthos). Protoplasts from each sample were burst in 1.5ml 100mM phosphate buffer ( $\text{Na}_2\text{HPO}_4$  +  $\text{KH}_2\text{PO}_4$ ) pH 7.0 and 3 replicates of 120 $\mu\text{l}$  used per assay. 20 $\mu\text{l}$  was removed from each protoplast sample for protein estimation. To the 120 $\mu\text{l}$  burst protoplasts in each microtitre plate well was added 120 $\mu\text{l}$  of substrate and stain (3mM acetylthiocholine iodide (ATCHI) and 1mM 5:5-dithiobis-2-nitrobenzoic acid (DTNB). The kinetics of the formation of the yellow anion were measured at 405nm on a Vmax microtitre plate reader for 30 cycles of 20 sec. 0.035 Units of AChE from *Torpedo marmorata* (Sigma) were used for a positive control, the 120 $\mu\text{l}$  of burst protoplasts being replaced by 120 $\mu\text{l}$  of phosphate buffer.

Inhibition of AChE using insecticide was studied by the addition of 10 $\mu\text{l}$  of a 0.25M solution of Bendiocarb (Bayer) in acetone.

Assays conducted in the presence of Triton X-100 included detergent at a concentration of 0.1% v/v in the protoplast lysis buffer and at 0.1% v/v in microtitre plate wells.

Phosphate buffer in place of lysate was used as the blank and experimental measurements adjusted accordingly from a mean value.

#### 2.22.5. Sabine Assay (Sabine, 1955).

The Sabine method, which is an extension of the assay developed by Hestrin (1949), was modified for use with the yeast system. The method is based on the reaction of acetylcholine with hydroxylamine to form acethydroxamic acid which forms a reddish brown coloured complex with ferric ions. Protoplast pellets were resuspended in 0.5ml of 67mM phosphate buffer pH 7.0 and, following bursting, 2 $\mu$ l was removed for protein estimation and 0.5ml of 8mM acetylcholineiodide (ACHI) in 67mM phosphate buffer added to each lysate. A negative control in which 0.5ml of 67mM phosphate buffer replaced the protoplasts and a blank in which 0.5ml of 67mM phosphate buffer replaced buffer containing substrate were also prepared. The samples were incubated at 30°C for 45 min. The reaction was terminated by the addition of 100 $\mu$ l of 20% trichloroacetic acid (TCA) and any remaining debris removed by microfuging for 1 min (13000 rpm). 0.5ml of each supernatant was transferred to clean 10ml plastic test tubes on ice and the concentration of substrate remaining in each one determined by the Sabine method: 1ml of freshly prepared alkaline hydroxylamine (equal volumes of 3.5N NaOH and 2M hydroxylamine hydrochloride mixed just prior to use) was added to each tube and placed at room temperature for 2 min. 0.5ml of HCl (a 1:3 dilution of concentrated HCl in distilled water) was added, followed by 0.5ml of ferric chloride (10% w/v in 0.1N HCl). The optical density of these solutions was read at 540nm (Cecil spectrophotometer) and the decrease in substrate concentration calculated by subtraction of the optical density reading of each sample from the reading of the negative control.



## 2.22.6. Standard Curves

### 2.22.6.a. Protein

A standard curve of OD<sub>600</sub> against known concentrations of protein was prepared using the BioRad detection system. 6ml of BioRad reagent was mixed with 24ml of SDW and 30 aliquots of 1ml pipetted into clean plastic test tubes on ice. A 10mg/ml solution of BSA in SDW was prepared. A range of solutions in triplicate containing between 1 and 10µg of BSA was produced by adding the required quantity of BSA from the stock solution to the test tubes. The tubes were shaken and the colour allowed to develop for 10 min. Solutions were read at OD<sub>600</sub> on a spectrophotometer (Cecil) and a standard curve plotted. The BSA stock solution was kept at -20°C.

### 2.22.6.b. ACHI

For the Sabine assay it was necessary to prepare a standard curve of OD<sub>540</sub> against known concentrations of ACHI in order to calculate the number of nmoles of ACHI hydrolysed. Concentrations of ACHI ranging from 0 - 8mM in 67mM phosphate buffer pH 7.0 were prepared in triplicate, assayed by the Sabine method, and read at OD<sub>540</sub>.

## 2.23. Inhibition of DAcHE from *S. cerevisiae* with Phenylmethylsulfonyl fluoride (PMSF)

Three 150ml cultures of yeast, one containing the gene for DAcHE in plasmid pG3 in the correct orientation (BJ2168R) and two with the gene in plasmid pG3 in the incorrect orientation (BJ2168W), were grown, split, and processed independently (2.22.1). Three replicates of BJ2168R and BJ2168W lysed protoplasts were assayed by the Ellman method (2.22.4) in the presence of 0mM, 1mM, 2mM, and 4mM final concentrations of PMSF. To the three additional replicates

of BJ2168W were added 0.035 Units of *Torpedo* AChE. The purpose of using pure AChE in the presence of BJ2168W was to allow for the presence of other proteins in the yeast lysates which may have decreased the amount of PMSF available for inhibition of the heterologously expressed AChE. The percentage inhibitions for the pure and heterologously expressed enzyme were calculated.

#### 2.24. Discontinuous Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis of Crude Yeast Extracts (after Hames, 1981)

SDS-PAGE analysis of crude protoplast extracts of BJ2168R was undertaken in order to visualize the recombinant DACH<sub>E</sub>. Protoplasts from cultures of BJ2168R and BJ2168W were prepared and pelleted as in 2.22.3. Protoplasts were lysed in 0.5ml of Protein Extraction Buffer (100mM Tris-HCl pH 6.8, 2% w/v SDS, 2mM EDTA) and 5 $\mu$ l taken for protein measurement (2.22.6.a). 50 $\mu$ l aliquots of lysed protoplasts were stored at -20°C until required.

A 7.5% resolving gel was prepared as follows:

SDW	19.18 ml
30% Acrylamide mix (29:1) (Sigma)	9.96 ml
1.5M Tris-HCl pH 8.8	10.0 ml
10% w/v SDS	400 $\mu$ l
Total volume	40.0 ml

The mixture was degassed for 15 min under vacuum. 400  $\mu$ l of 10% w/v ammonium persulphate and 13.4  $\mu$ l TEMED (Tetramethylethylenediamine) were added and the gel quickly poured. Water saturated 1-butanol was layered over the top of the gel using a Pasteur pipette and the gel left to polymerize (at this stage the gel could be left at 4°C overnight and the stacking gel poured the following day if



required).

A 4% stacking gel was prepared as follows:

SDW	14.66 ml
30% Acrylamide mix (29:1) (Sigma)	2.66 ml
1M Tris-HCl pH 6.8	2.50 ml
10% w/v SDS	200 $\mu$ l

The mixture was degassed for 10 min under vacuum.

600  $\mu$ l of 10% w/v ammonium persulphate and 20  $\mu$ l of TEMED were then added and the stacking gel quickly poured. The comb was carefully placed taking care to avoid bubbles and the gel left to polymerize.

The gel running buffer was: 25mM Tris-HCl pH 8.3, 250mM glycine, 0.1% w/v SDS freshly made from a 5X stock.

The sample loading buffer was: 100mM Tris-HCl pH 6.8, 2% w/v SDS, 0.2% bromophenol blue, 20% v/v glycerol.

To a protein sample of 20 $\mu$ l in an Eppendorph tube was added 20 $\mu$ l of loading buffer and 4 $\mu$ l 1M Dithiothreitol (DTT). The top of the Eppendorph was then pierced and the sample placed in a boiling water bath for 10 min. On removal from the boiling water bath the sample was spun for 10 sec in a microfuge (13000 rpm) and placed on ice until loading.

The comb was carefully removed from the stacking gel and the running buffer poured into the electrophoresis apparatus (Biorad Protean II). Samples were loaded using fine Gilson pipette tips and then electrophoresed at 110V for 7 hr at RmT°C.

## 2.25. Silver Staining of Proteins

The gel was fixed overnight in a solution of 40% v/v

ethanol, 10% v/v acetic acid. It was rinsed with 20% v/v ethanol for 10 min, then in SDW for 10 min, sensitized with sodium thiosulphate pentahydrate (0.03% w/v) for 1 min, and rinsed with SDW (2X 1 min). Silver nitrate solution was added (0.2% w/v  $\text{AgNO}_3$ , 0.025% v/v 12.3M formaldehyde solution) and staining allowed to proceed for 45 min. The gel was rinsed briefly in SDW (10-20 sec) and then developed with sodium carbonate solution (3% w/v  $\text{Na}_2\text{CO}_3$ , 0.025% v/v 12.3M formaldehyde solution, 0.001% w/v sodium thiosulphate pentahydrate) until optimal resolution was obtained. The reaction was stopped by soaking the gel in a solution containing 5% w/v Tris, 2% v/v acetic acid for 30 min. The gel was then stored in a heat sealed plastic bag containing 2-3 ml SDW.

#### **2.26.a. Extraction of Total RNA from Yeast Cells containing the DACH<sub>E</sub> Gene in pBM150 to Investigate Transcriptional Expression of DACH<sub>E</sub> (after Schuler and Zielinski (1989))**

Total RNA was isolated from 50ml cultures of transformed and non-transformed cells of EJL363-6D grown in YPD medium (2.7) to an  $\text{OD}_{600}$  of 1.0 (mid-exponential phase). Transformants of EJL363-6D containing the gene under the galactose promoter in pBM150 were grown to an  $\text{OD}_{600}$  of 0.5, harvested, washed with galactose YPD (2% w/v galactose in place of 2% w/v glucose), and induced in 50ml galactose YPD to an  $\text{OD}_{600}$  of 1.0. Normal sterile DNA procedures were employed throughout. All solutions were autoclaved before use with the exception of 8M urea which was made up with SDW.

Cells were harvested in sterile glass Corex tubes by centrifugation at 4°C, 3200 rpm for 10 min (MSE Mistral). To each cell pellet were added 0.3g glass beads (50 mesh BDH, acid washed), 8ml 20mM Tris-HCl pH 8.5, 10mM EDTA, 1% w/v SDS, 0.2M NaCl, and 8ml phenol:chloroform:isoamyl alcohol (50:49:1 v/v/v). Phenol was equilibrated with 20mM



Tris-HCl pH 8.5, 10mM EDTA, 1% w/v SDS according to the method of Maniatis et al (1982). The cell suspension was vortexed for 3 min at room temperature. Aqueous and organic phases were separated by centrifugation at 6000 rpm for 20 min (Beckman J2-21) at 4°C. The upper aqueous phase was transferred to a fresh sterile Corex tube and 8ml chloroform:isoamyl alcohol (49:1 v/v) added. The tube was then vortexed for 10 min. The upper aqueous phase was transferred to a fresh sterile tube and the previous extraction repeated. Nucleic acids were precipitated by the addition of 8ml IPA and leaving at -20°C for 1 hr. Precipitated nucleic acids were collected by centrifugation at 6000 rpm for 20 min at 4°C. The supernatant was discarded and the pellet allowed to air dry for 5 min. Nucleic acids were reprecipitated by resuspending the pellet in 5ml of TE (2.8.1), adding 200 $\mu$ l 5M NaCl and 12.5ml ice cold absolute EtOH, and incubating in ice for 20 min. Following centrifugation at 6000 rpm for 20 min at 4°C the pellet was resuspended in 1ml of TE and transferred to a sterile Eppendorph tube. High molecular weight RNA was precipitated by adding 0.25 vol of 8M Urea followed by 0.25 vol 10M LiCl. The tube was incubated overnight in an ice water bath. The RNA was collected by microfuging at 4°C (13000 rpm, 15 min) and resuspended in 500 $\mu$ l TE. The RNA was reprecipitated by the addition of 50 $\mu$ l 3M KOAc pH 4.8 and 1ml ice cold absolute EtOH and leaving at -20°C for 1 hr. RNA was collected by microfuging at 4°C (13000 rpm, 15 min), allowed to air dry, and resuspended in TE.

#### **2.26.b. Extraction of Total RNA from Yeast Cells containing the DAcHE Gene in pG3 to Investigate Transcriptional Expression of DAcHE (Qiagen RNeasy Method)**

Total RNA was extracted at different stages of the growth curve from M.M cultures of yeast cells containing the DAcHE gene in pG3. The medium was supplemented with the appropriate amino acids and the cultures split into two and

processed independently. For each stage a suitable volume (based on the OD<sub>600</sub> reading) of culture was sampled to provide  $\approx 5 \times 10^7$  cells for RNA extraction. Cells were harvested and protoplasted as in 2.9 except that in the case of lag phase and stationary phase cells 4X the concentration of Zymolyase was used in the SZB buffer.

Total RNA was extracted from pelleted protoplasts using Qiagen columns in accordance with the manufacturer's instructions. For each sample protoplasts were transferred to Eppendorph tubes and lysed in 350 $\mu$ l of RLT buffer containing 4M guanadine thiocyanate.  $\beta$ -mecaptoethanol at 0.1% v/v was added to buffer RLT (supplied) immediately prior to use. The lysate was microfuged (13000 rpm MSE) for 2 min and the supernatant transferred to a fresh Eppendorph tube. 1 vol of 70% v/v ethanol was added and mixed well with the lysate by pipetting. The sample was applied to an RNeasy minispin column sitting in a 2ml collection tube (supplied) and microfuged for 10 sec at 13000 rpm. The flowthrough was discarded. The column was then washed in accordance with the manufacturer's instructions with high salt and low salt containing ethanol buffers (supplied) which remove contaminating proteins, DNA and reduce low molecular weight RNAs in the final total RNA preparation. Total RNA was eluted into a fresh collection tube by pipetting 50 $\mu$ l of RNase free water (supplied) directly onto the membrane in the column, leaving for 10 min, before microfuging for 10 sec at 13000 rpm.

## 2.27. Quantification of RNA

This was exactly as described for DNA (2.10) except that an OD of 1 corresponds to approximately 40 $\mu$ g/ml for RNA and the A<sub>260</sub>/A<sub>280</sub> ratio should be 2.0 for a pure preparation of RNA.



## 2.28. Denaturing Agarose Gel Electrophoresis of RNA

The denaturant used was formaldehyde. RNA was electrophoresed in 1.1% w/v agarose gels made up in 1X gel running buffer (5X: 0.2M morpholinopropanesulphonic acid (MOPS) pH 7.0, 50mM NaOAc, 5mM EDTA pH 8.0) containing 2.2M formaldehyde. Agarose was melted in SDW, cooled to 60°C, and 5X gel buffer and formaldehyde added to give the required concentrations. ALL GELS WERE POURED AND RUN IN A FUME HOOD DUE TO THE TOXIC NATURE OF FORMALDEHYDE. RNA samples were prepared as follows:

RNA (up to 20µg)	4.5 µl
5X gel buffer	2.0 µl
Formaldehyde	3.5 µl
Formamide	10.0 µl

Formamide was deionized by batch mixing with a mixed bed resin (BioRad AG 501-X8) until its pH was neutral. The RNA samples were then incubated at 55°C for 15 min, 2µl of sterile loading buffer (50% v/v glycerol, 1mM EDTA, 0.4% w/v bromophenol blue) added, and gels run at 20mA in 1X gel running buffer. RNA size markers as used in 6.3.6 were RNA molecular weight marker I (Boehringer Mannheim) at a concentration of 2µg/µl and 10µl were added to the appropriate volume of sample buffer and loading buffer before denaturation. Gels were stained after electrophoresis with ethidium bromide (0.1µg/ml for 30 min) and examined under UV illumination. Fluorescence was recorded using a computer camera (Mitsubishi).

## 2.29. Northern Blotting

The blotting and subsequent fixation of RNA onto nylon membrane (Biodyne A (Pall Biosupport) or Hybond N+ (Amersham)) (after Thomas, 1980) was performed exactly as described for Southern blotting of DNA (2.18.) with the

omission of the denaturation and neutralization steps that are carried out for DNA after separation by agarose gel electrophoresis.

### **2.30. Northern Hybridization of RNA with Radiolabelled DNA Probes.**

Prehybridization, hybridization, and radiolabelling of probes exactly as described for DNA (2.21.).

### **2.31. Calculation of Specific Activity of Radiolabelled DNA Probes by Differential Precipitation with Trichloroacetic Acid (TCA).**

5 $\mu$ l samples of each probe to be assayed were carefully spotted onto the centre of a pair of Whatman GF/C glass fibre filters suitably marked with a pencil. The filters were allowed to dry. Using forceps one of each pair of filters was transferred to a glass beaker containing 200 ml of ice cold 5% v/v TCA, 20mM sodium pyrophosphate. The filter was swirled in the TCA solution for 2-3 min and then transferred to a second beaker containing the same volume of ice cold TCA solution. The wash was repeated a further two times before transferring the filter briefly to a fresh beaker containing 200 ml 70% v/v ethanol. The filter was allowed to air dry. Each of the filters for each probe (washed and unwashed) was then placed in a scintillation vial and the amount of radioactivity measured using a liquid scintillation counter.

### **2.32. The Generation of a Homologous cDNA Probe from *Culex Molestus***

RT-PCR was employed in the generation of a homologous cDNA fragment from *Culex molestus* mRNA that could in the future be used to isolate the AChE gene from a *Cx. molestus* cDNA library. The efficient synthesis of a cDNA from the mRNA



template is dependent upon the isolation of high quality total RNA which then retains its quality through the mRNA purification procedure. Stringent precautions were necessary to minimize RNase activity both during the initial stages of extraction and subsequently when the RNA has been purified. The following precautions were employed:

1) All reagents used were bought fresh, guaranteed RNase free from Sigma.

2) All solutions (except GTME) were treated overnight with 0.1% v/v diethylpyrocarbonate (DEPC), a strong inhibitor of RNase and then autoclaved to remove traces of DEPC. DEPC is highly toxic and solutions were treated in a fume hood.

3) Tris buffer, which cannot be directly treated with DEPC, was made up with RNase free Tris and DEPC treated SDW, then autoclaved to remove traces of DEPC.

4) Items of glassware and plasticware to be used in handling RNA work were set aside and used only for RNA work.

5) Wherever possible sterile, disposable plasticware (which is essentially free of RNase) was used for the preparation and storage of RNA. Any plasticware that was not available presterilized was autoclaved twice.

6) All glassware, spatulas, homogenizers, mortars and pestles, were treated by baking at 200°C for 6 hr.

7) Disposable plastic gloves were worn for all manipulations involving RNA and were changed frequently.

8) Ultracentrifuge tubes were treated directly with 0.1% DEPC in SDW and then autoclaved.

9) The powerful chaotropic agent guanidine thiocyanate was used as an inhibitor of endogenous ribonucleases during the initial stages of extraction.

10) Vanadyl-ribonucleoside complexes (Sigma) were used to protect RNA once it had been isolated. These are transition state analogues that bind to RNase and inhibit its activity.

#### 2.32.1. Isolation of Total RNA from *Cx. molestus*

Total RNA was prepared from a batch of 150 pupae (between 1 and 3 days old) that had been stored in liquid nitrogen. The insects were removed from the cryotube and ground in liquid nitrogen using a mortar and pestle. The ground tissue was then quickly transferred to a glass homogenizer, thawed out into 10ml GTME solution (4M guanidine thiocyanate, 25mM sodium citrate, 0.5% w/v sarcosine, 1% v/v  $\beta$ -mecaptoethanol), and homogenized on ice for 5 min.

The lysate was transferred to a 50ml Falcon tube and a further 10ml GTME added. Cellular debris was removed by centrifuging at 3200 rpm for 5 min (MSE Centaur 2). The supernatant was transferred to a glass beaker and the DNA sheared by passing the lysate through a 19G X 2" sterile needle attached to a 20ml sterile syringe (at least 20 passes). The lysate was transferred to a fresh 50ml Falcon tube and the remaining debris pelleted by centrifuging at 3200 rpm for 15 min. The tube was left on ice while 2 X 9.5ml 'cushions' of caesium chloride (5.7M in 0.1M EDTA pH 7.5) were prepared in 2 DEPC treated polycarbonate Beckman centrifuge tubes. Using a sterile 10ml pipette 10ml of homogenate was loaded onto each caesium chloride cushion and each tube then filled to the top with GTME solution. The tubes were balanced to within 0.1g and ultracentrifuged at 27000 rpm for 28 hr (Beckman L5-50 SW28 rotor).



The bulk of the supernatant was aspirated off from each tube, stopping approximately 1cm from the bottom or immediately after removal of the DNA band. The remainder was quickly decanted off, taking care not to dislodge the RNA pellet, and the tubes kept inverted on sterile tissue paper for 5 min. The bottom of each tube was cut off using a baked razor blade, retaining enough of the tube to form a small 'cup'. The cups were placed on ice. The RNA was resuspended in 250 $\mu$ l TE (2.8.1) (pH 7.4) with repeated pipetting, transferred to an Eppendorph tube, and vortexed thoroughly. It was heated to 65°C for 10 min and revortexed. The RNA solution was made up to 500 $\mu$ l with TE and extracted with an equal volume of chloroform:butanol (1:1). The aqueous layer was removed and the RNA precipitated with 1/10 vol 0.3M NaOAc (pH 5.3) and 2 vol absolute EtOH. Precipitation was overnight at -20°C. The RNA pellets were retrieved by microfuging (13000 rpm) for 15 min and then resuspended in 250 $\mu$ l DEPC treated water. The concentration of RNA was measured spectrophotometrically. Vanadyl-ribonucleoside complexes were added to 10mM and the RNA stored at -70°C.

#### 2.32.2. Non-denaturing Agarose Gel Electrophoresis of RNA

Non-denaturing agarose gel electrophoresis was used to determine the quality of isolated RNA. A non-denaturing 1.2% w/v TBE agarose gel was used. The agarose was made up and the gel cast exactly as described for DNA (2.12.) but the buffer used was TBE (89mM Tris-Borate, 2mM EDTA). RNA was electrophoresed alongside DNA size markers for 20 min at 5V/cm and the gel stained with ethidium bromide (0.1 $\mu$ g/ml in 1 X TBE) for 15 min. RNA was visualized on a UV transilluminator and a photograph taken using a computer camera.

### 2.32.3. Purification of Poly A+ RNA from Total RNA

Poly A+ RNA was purified from total RNA by means of Hybond-mAP messenger affinity paper (Amersham). Approximately 10 $\mu$ g of poly A+ mRNA can be isolated per cm<sup>2</sup> Hybond-mAP. For isolation purposes the percentage of mRNA in total RNA was assumed to be 2%. Hybond-mAP was cut into squares of 1cm<sup>2</sup> and the squares pre-wetted with 20 $\mu$ l 0.5M NaCl (0.5M NaCl treated with 0.1% DEPC and autoclaved) and allowed to air dry on sterile filter paper. A 40 $\mu$ l aliquot containing approximately 400 $\mu$ g of total RNA in DEPC treated water was heated at 65°C for 5 min to denature the RNA and immediately chilled on ice. 10 $\mu$ l of 2M DEPC treated NaCl was then added. A square of Hybond-mAP was placed on Parafilm and the total RNA solution SLOWLY applied to the paper in 2 $\mu$ l aliquots. The paper was allowed to dry between each aliquot. When all of the total RNA had been applied the paper was left on Parafilm for 5 min and transferred to sterile filter paper. Any RNA solution remaining on the Parafilm was re-spotted onto the paper. The paper was left to dry for 5 min on the sterile filter paper. The square was transferred to a 15ml Falcon tube and washed in 5ml of 0.5M NaCl for 5 min. This step was repeated with fresh 0.5M NaCl. The 0.5M NaCl was discarded and the square washed in 5ml 70% v/v EtOH (made up with DEPC treated water) with shaking for 2 min. This step was repeated. The square was transferred to sterile filter paper and allowed to air dry for 10 min. The square was cut into smaller fragments and transferred to a sterile Eppendorph tube. 40 $\mu$ l of DEPC treated water was added and the Eppendorph tube heated at 70°C for 5 min to release the mRNA into solution. The tube was briefly vortexed and the mRNA solution transferred to a fresh sterile Eppendorph using a Gilson pipette. Vanadyl-ribonucleoside complexes were added to 10mM and the mRNA stored at -70°C.



#### 2.32.4. RT-PCR

Primers were designed based on the partial genomic sequence available for the *Cx. pipiens* AChE gene (Rooker, 1994). Three sets of homologous primers each 20 bases long (Genosys) were synthesised: KS<sub>1</sub> (specific for a sequence in exon 2) and KS<sub>2</sub> and KS<sub>3</sub> (specific for sequences in exon 3). KS<sub>1</sub> and KS<sub>2</sub> were both forward primers and KS<sub>3</sub> the reverse.

##### 2.32.4.a. Reverse Transcription

This part of the RT-PCR was based on the Amersham protocol for first strand cDNA synthesis. 1µg purified mRNA in a volume of 2µl DEPC treated water (10mM vanadyl-ribonucleoside complexes) was used per reaction. The mRNA was heated to 65°C for 10 min (to remove secondary structure) and then cooled on ice before adding to the RT mix. The reaction was set up on ice as follows:

5X RT Buffer	4 µl
Sodium pyrophosphate	1 µl
Human Placental Ribonuclease Inhibitor	1 µl
Deoxynucleoside Triphosphate (dNTPs) Mix	2 µl
Oligo dT	1 µl
<i>Cx. molestus</i> mRNA	2 µl
DEPC Treated Water	8 µl
Reverse Transcriptase (20 Units)	1 µl
Final Volume	20 µl

The reaction was incubated for 1 hr at 42°C.

##### 2.32.4.b. PCR (after Innis et al, 1990)

The 20µl reaction volume was made up to 500µl with SDW and 5µl of this used per PCR reaction (Jones, pers.comm., 1994). 4 PCR reactions were set up at a range of final

MgCl<sub>2</sub> concentrations (1 - 4mM) in duplicate on ice and components added in the following order:

SDW	72, 70, 68, or 66 $\mu$ l
10X PCR Buffer (Gibco-BRL)	10 $\mu$ l
dNTP Mix (2 $\mu$ l each of 10mM dATP, dTTP, dGTP, dCTP)	8 $\mu$ l
KS <sub>2</sub> or Oligo dT	1 $\mu$ l
KS <sub>1</sub> or KS <sub>3</sub>	1 $\mu$ l
Taq Polymerase (5 Units) (Gibco-BRL)	1 $\mu$ l
From RT Reaction	5 $\mu$ l
MgCl <sub>2</sub> (50mM Stock)	2, 4, 6 or 8 $\mu$ l
Final Volume	100 $\mu$ l

The components were mixed well using a Gilson pipette, briefly spun in a microfuge (13000rpm) to eliminate any air bubbles, and reactions overlaid with 100 $\mu$ l molecular biology grade mineral oil (Sigma). Controls were set up at each MgCl<sub>2</sub> concentration and contained 5 $\mu$ l SDW instead of 5 $\mu$ l from the RT reaction. PCR cycling was performed on a Hybaid computer controlled heating block as follows:

Initial Melt:	95°C	5 min.
Then for 45 Cycles*:		
	95°C	1 min.
	45°C	1 min.
	72°C	1 min.
Then Final Extension:	72°C	8 min.

\* After 15 cycles a further 5 Units of Taq Polymerase were added into each reaction.

RT-PCR fragments obtained were then further amplified by cycling at the same temperatures for 35 cycles (no additional Taq was added). 1 $\mu$ l of RT-PCR reaction mix was used for reamplification at the appropriate MgCl<sub>2</sub> concentration.



## CHAPTER THREE ANALYSIS OF DACH<sub>E</sub> EXPRESSION FROM CONSTRUCT pBM150-DACH<sub>E</sub>

### 3.1. Introduction

Initial studies centred on the analysis of an expression construct already available within the laboratory: pBM150-DACH<sub>E</sub>. This construct was made by a *Sma*I-*Hinc*II excision of the *D. melanogaster* AChE cDNA from Bluescript KS+ (Hall and Spierer, 1986), ligation of *Eco*RI linkers (the endogenous *Eco*RI site having been previously methylated), and subsequent insertion into the *Eco*RI site of the shuttle vector pBM150 to create pBM150-DACH<sub>E</sub>. pBM150-DACH<sub>E</sub> was then electroporated into the *S. cerevisiae* strain EJL363-6D (Curran, pers.comm, 1991). pBM150 is an inducible single copy centromeric plasmid which utilizes the yeast *GAL10* promoter for the expression of heterologous genes.

Preliminary analyses were carried out using the assay developed by Ellman et al for the detection of AChE. Since expression from pBM150 is intracellular, breakage of the yeast cell wall was necessary in order to release enzyme. An unacceptable level of interference in the assay was encountered on breakage which made an assessment of AChE expression virtually impossible. The cause of this interference was investigated and found to be thiol groups within the *S. cerevisiae* cell wall reacting with the dithiobisnitrobenzoate ion of the assay reaction.

Subsequent experiments with protoplasts indicated that active AChE was not being expressed from pBM150-DACH<sub>E</sub> or being expressed at undetectable levels. Total RNA was extracted from *S. cerevisiae* containing pBM150-DACH<sub>E</sub> and Northern blots carried out to determine whether transcription of the gene was occurring.

### 3.2. Materials and Methods

Culturing of transformed and non-transformed cells for expression, harvesting, and cell wall breakage by the glass bead method were as described in 2.22.1 and 2.22.2. Final resuspension was in 200 $\mu$ l of 1% v/v Triton X-100 0.1M phosphate buffer pH 7.0 (TPB), followed by brief vortexing to mix, before microfuging (13000 rpm, 2 min, MSE). Mosquito homogenates for positive controls were prepared as follows: individual adults of *Cx. molestus* were homogenized on ice in 200 $\mu$ l of TPB in an Eppendorph tube and spun for 5 min in a pre-cooled microfuge (13000 rpm) to remove debris. Supernatants were then used directly in the assay.

Each microtitre plate well contained: 135 $\mu$ l 0.1M TPB, 10 $\mu$ l 10mM DTNB in 0.1M phosphate buffer pH 7.0, 30 $\mu$ l sample, to which was added 25 $\mu$ l of the substrate 10mM ATCHI in SDW at the start of the assay. The kinetics of the formation of the yellow anion (5-thio-2-nitrobenzoic acid) were measured at 405nm on a V max microtitre plate reader for 30 cycles of 20 sec.

The cells to be tested were divided into 4 groups:

- 1) Transformed cells grown initially on glucose and then induced on galactose.
- 2) Transformed cells grown on glucose.
- 3) Non-transformed cells of the same strain grown initially on glucose and then on galactose.
- 4) Non-transformed cells grown on glucose.

In addition 3 or 4 replicates of a mosquito homogenate were also used as a positive control.

The yeast strain EJL363-6B (untransformed) was used throughout investigations into the cause of *S. cerevisiae* interference in the Ellman assay and cells from 50ml M.M.



(2.2) cultures were harvested and broken as described in 2.22.2. Final resuspension was in 200 $\mu$ l 0.1M TPB, followed by brief vortexing to mix, before microfuging (13000 rpm, 2 min, MSE). Supernatants were diluted as required and 30 $\mu$ l aliquots then used in microtitre plate assays.

Protoplasts were prepared from 50ml YPD (2.7) cultures grown overnight at 27°C to an OD<sub>600</sub> of approximately 0.8. Cells were harvested at 3200 rpm (MSE Centaur 2), supernatants discarded, and pellets resuspended in 5ml 1M sorbitol. Zymolyase 20,000 was added at a concentration of 5mg/ml and samples incubated at 37°C for 45 min. Samples were gently mixed from time to time. Protoplast formation was confirmed by taking 100 $\mu$ l of sample and observing 'clearing' in 1ml of SDW. The protoplasts were harvested at 3200 rpm and washed each time with 1M sorbitol. Final resuspension was in 0.5ml 0.1M TPB to lyse and 30 $\mu$ l samples were used undiluted.

To digest the cell wall further Snail Helix Pomatia Juice (SHP) (IBF Biotechnics) was added at the Zymolyase step (100 $\mu$ l for 50ml cells) in the presence of the disulphide reducer dithiothreitol (DTT) at 10mM.

Proteinase K was added to experimental samples at a concentration of 200 $\mu$ g/ml and incubated at 37°C for 1 hr with suitable controls. Samples were tested, with and without substrate, in the Ellman assay on a microtitre plate reader at 405nm and endpoints recorded.

The spun columns used were prepared as described in 2.21.2. The column was rinsed with 0.1M TPB. 30 $\mu$ l undiluted *S. cerevisiae* sample supernatant after cell breakage was added to 270 $\mu$ l 0.1M TPB and spun through the column at 3000rpm (MSE Centaur 2) for 5 min. Samples were collected in 15ml disposable plastic tubes.

Culturing of transformed and untransformed cells, harvesting, and protoplasting procedures for analysis of expression from protoplasts were as described in 2.22.1. and 2.22.3. Experimental and control groups were as described above. Protoplast microtitre plate assays by the method of Ellman were as described in 2.22.4.

Total RNA was isolated from transformed cells that had been induced on galactose and from suitable controls as in 2.26.a. Electrophoresis of total RNA was by the formaldehyde method (2.28).

Northern blotting was performed using Biodyne A nylon membranes as in 2.29. Hybridization of membranes was adapted from Wahl et al (1979) (2.30).

A *Bam*HI digest of pKS-DAC<sub>H</sub>E produced a 1.5 Kb fragment containing a segment of the DAC<sub>H</sub>E cDNA that was purified, labelled with <sup>32</sup>P, and used as a probe for Northern blots (2.21.2).

A *S. cerevisiae* ribosomal probe was also prepared to check the fidelity of Northern blotting procedures. Plasmid pY1rA12 (Petes et al, 1978) (Figure 3.1) containing the yeast ribosomal genes was transformed into *E. coli* and prepared as in 2.6 and 2.8.1. Digestion of pY1rA12 with *Hind*III yielded the following fragment sizes: 0.98, 2.46, 5.05, 6.11 Kb. The 6.1 Kb fragment was used to make a radiolabelled probe and hybridized to Northern blots.

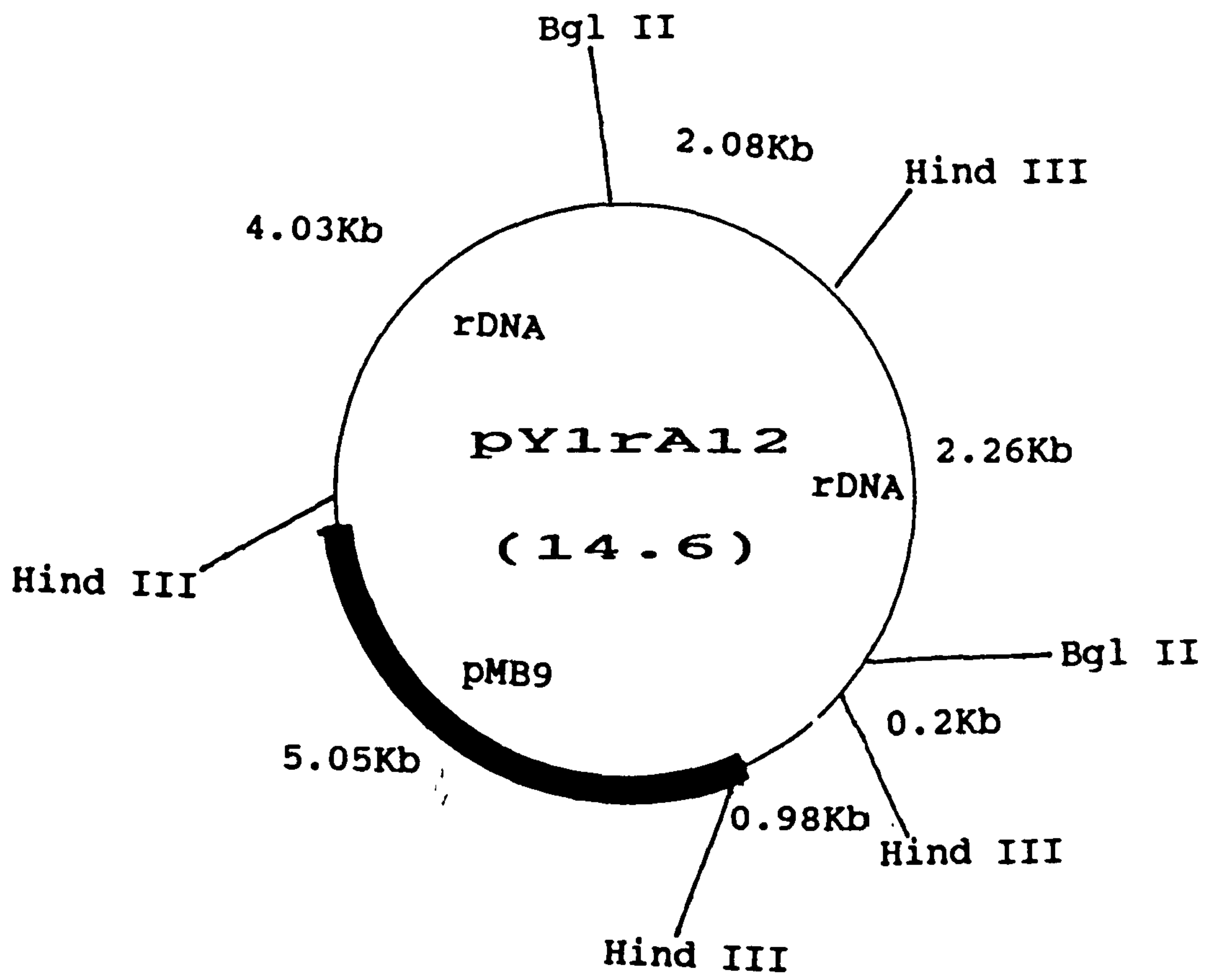
### 3.3. Results

#### 3.3.1. Preliminary Analysis of Expression

It was impossible to test for AChE activity using the Ellman assay with *S. cerevisiae* since a cell breakage



Figure 3.1. Plasmid pYlrA12. The plasmid contains the entire rDNA locus encompassed by the original seven *EcoRI* fragments derived from *S.cerevisiae* rDNA described by Petes *et al* (1978).





percentage of between 5 and 10% (confirmed by examination of cells under the microscope) resulted in a background interference of >3.5 OD units (beyond the range of the microtitre plate reader). The same reading 'over' was obtained for all four groups of cells whenever tested.

### **3.3.2. Investigations into the Cause of Interference by *S. cerevisiae* in the Ellman Assay**

An observation that intact yeast cells left overnight in microtitre plate wells in the presence of the assay reagents produced only a slight yellow colour suggested that it was disruption of the cell wall that led to background interference (data not shown).

Several experiments were done to test the hypothesis that it was thiol groups released from the yeast cell wall on breakage that were responsible for the interference: 1) cell debris and supernatants were tested; 2) protoplasts were made and washed a different number of times before testing; 3) Proteinase K was employed to eliminate the possibility of an endogenous enzyme peculiar to *S. cerevisiae* being responsible for breaking down the substrate.

See Appendices 1.1 - 1.5. for tabulated data showing means +/- S.E.M. for all experiments, together with tests of significance by Student's *t*.

#### **3.3.2.a. Cell Wall Interference by *S. cerevisiae* in the Ellman Assay Examined by Incubation with Proteinase K**

Substrate hydrolysis by an endogenous yeast esterase may have been the cause of the interference observed. If this were the case then incubation with Proteinase K might have been expected to disrupt its activity. This was not found to be the case (Figure 3.2.). It can be seen that

incubation with Proteinase K had very little effect upon the level of interference (mean  $OD_{405}$ s of  $0.155 \pm 0.022$  and  $0.179 \pm 0.009$  for the presence and absence of Proteinase K respectively ( $p = >0.1$ ). Furthermore, absence of substrate (ATCHI) made no difference ( $p = >>0.1$  when Y-PK+S is compared to Y-S and  $p = >0.1$  when Y+PK+S is compared to Y-S). The observation that interference occurs independently of the substrate ATCHI is also strongly indicative of a non-enzymatic cause. In contrast the mosquito controls showed a marked difference in ability to utilize the substrate when incubated in the presence or absence of Proteinase K ( $p = <0.001$ ). Proteinase K virtually destroyed mosquito AChE catalytic activity to a level where it recorded an optical density barely above that of the blank (blank was all reagents without yeast or mosquito homogenate). It can also be seen that the presence of substrate was crucial to mosquito controls ( $p = <0.001$  when M-PK+S is compared to M-S).

### 3.3.2.b. Testing of Cell Pellet and Supernatant Fractions

It was not possible to accurately assess interference levels in the presence of the cell pellet fraction due to debris settling on the bottom of microtitre plate wells. However, an intense yellow colour was observed by eye when pellets from broken cells were incubated with the assay components. The colour observed was independent of the substrate ATCHI. Differential centrifugation of diluted supernatants made no significant difference ( $p = >0.1$  when the  $OD_{405}$  of a supernatant from a 3000 rpm spin for 5 min (MSE Centaur 2) is compared to that of a supernatant from a 13000 rpm spin for 10 min (MSE microfuge). Once again the presence or absence of substrate made no difference to the level of interference observed ( $p = >0.1$  when supernatants from 3000 rpm spins are compared + or - substrate;  $p = >>0.1$  when supernatants from 13000 rpm spins are compared + or - substrate) (Figure 3.3).

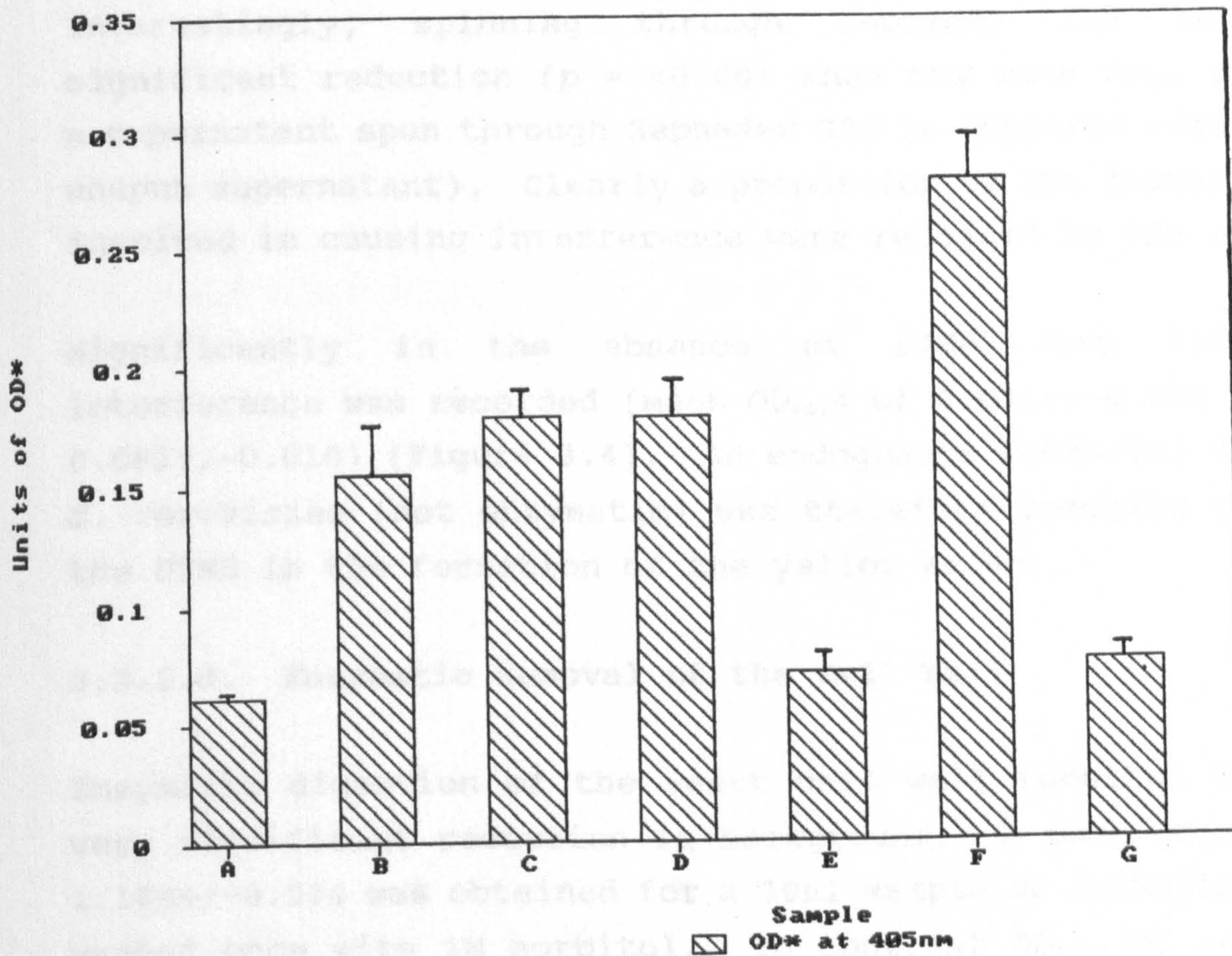


Figure 3.2. The effect of proteinase K on background interference by *S. cerevisiae* in the Ellman assay.

- A: Blank
- B: Yeast + PK + Substrate
- C: Yeast - PK + Substrate
- D: Yeast - Substrate
- E: Mosquito + PK + Substrate
- F: Mosquito - PK + Substrate
- G: Mosquito - Substrate

\* Units given are for raw absorbances since there was clearly no enzyme involved. Results are for 1 in 20 dilutions of yeast samples (200 $\mu$ l in 4ml 0.1M TPB) to bring the OD within the range of the plate reader.







### 3.3.2.c. Effect of Boiling, Use of a Spun Column, and Absence of DTNB

1 in 10 dilutions of *S. cerevisiae* sample 3000 rpm (MSE Centaur 2) supernatants were boiled for 5 min and this was found, if anything, to slightly increase the level of interference. This provided further evidence that the cause was non-enzymatic.

Interestingly, spinning through Sephadex led to a significant reduction ( $p = <0.001$  when the mean  $OD_{405}$  from a supernatant spun through Sephadex G25 is compared with an unspun supernatant). Clearly a proportion of the factor(s) involved in causing interference were retained by the gel.

Significantly in the absence of DTNB very little interference was recorded (mean  $OD_{405}$ s of  $0.071 \pm 0.006$  and  $0.081 \pm 0.010$ ) (Figure 3.4). An endogenous factor(s) from *S. cerevisiae* (not enzymatic) was therefore reacting with the DTNB in the formation of the yellow anion.

### 3.3.2.d. Enzymatic Removal of the Cell Wall

Enzymatic digestion of the yeast cell wall resulted in a very significant reduction in background. A mean  $OD_{405}$  of  $1.188 \pm 0.034$  was obtained for a  $30\mu\text{l}$  sample of protoplasts washed once with 1M sorbitol. In contrast  $OD_{405}$ s of  $>0.15$  were obtained during the Proteinase K experiments for 1 in 20 dilutions of yeast cells broken with glass beads. Furthermore, protoplasts were made from YPD cultures grown to an  $OD_{600}$  of 0.8 rather than M.M cultures grown to an  $OD_{600}$  of 0.1. Taking into account the volume of resuspension ( $0.5\text{ml}$  and  $0.2\text{ml}$  for protoplasts and glass bead extracts respectively) the number of protoplasts present was higher by approximately three fold.

Figure 3.3. A comparison between the level of interference caused by two supernatant fractions in the presence and in the absence of substrate. It was not possible to accurately assess the level of interference associated with the cell pellet fraction (see text).

- A: Supernatant (rpm 3000+) + Substrate
- B: Supernatant (rpm 3000+) - Substrate
- C: Supernatant (rpm 13000) + Substrate
- D: Supernatant (rpm 13000) - Substrate

\* Results are for 1' in 10 dilutions of yeast samples spun at 3000 rpm (MSE Centaur 2) and 13000 rpm (MSE microfuge).



Figure 1.4. The effect of boiling the supernatant on the OD of a spin column, and absence of DNA in the supernatant. Further investigate the cause of interference.

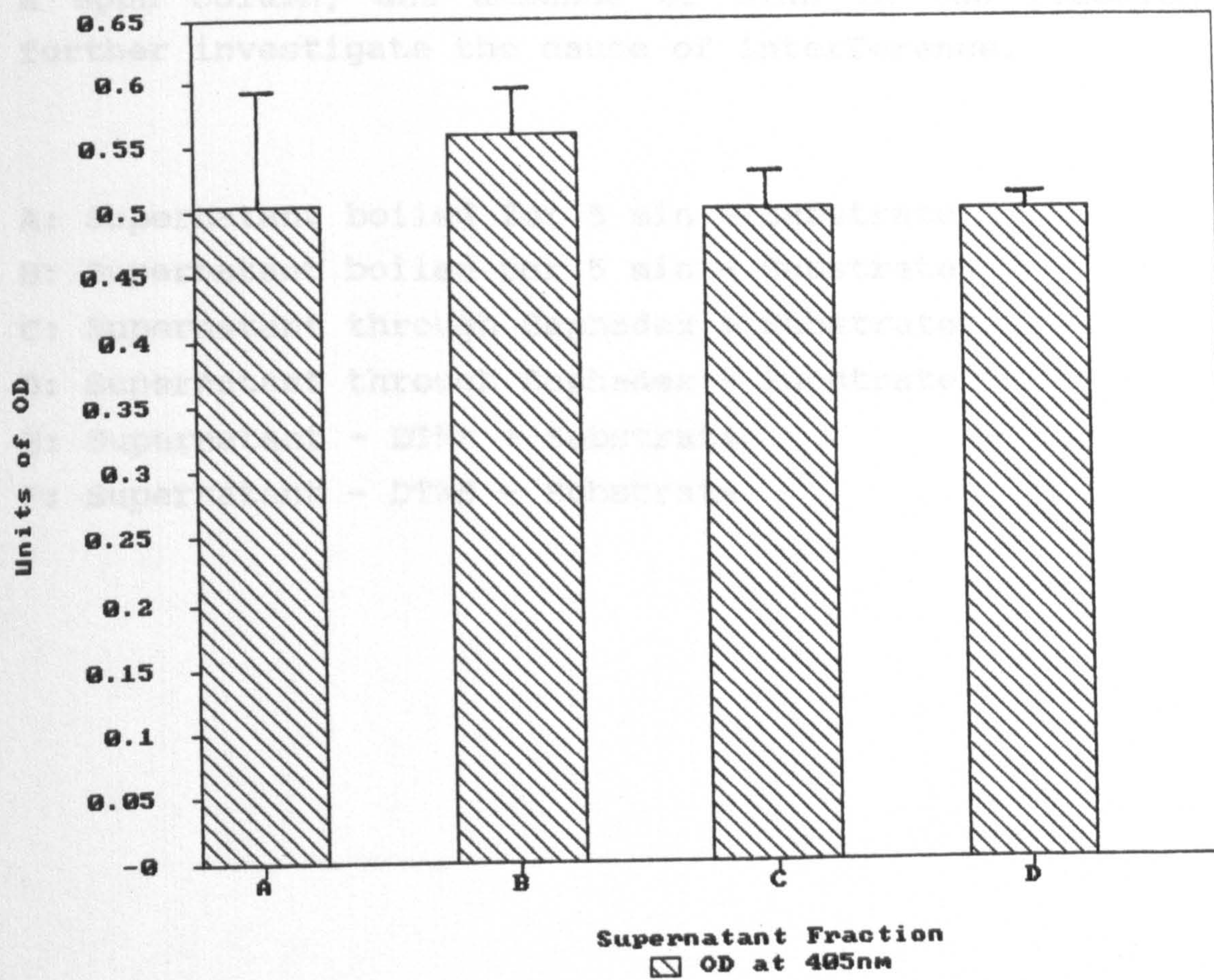
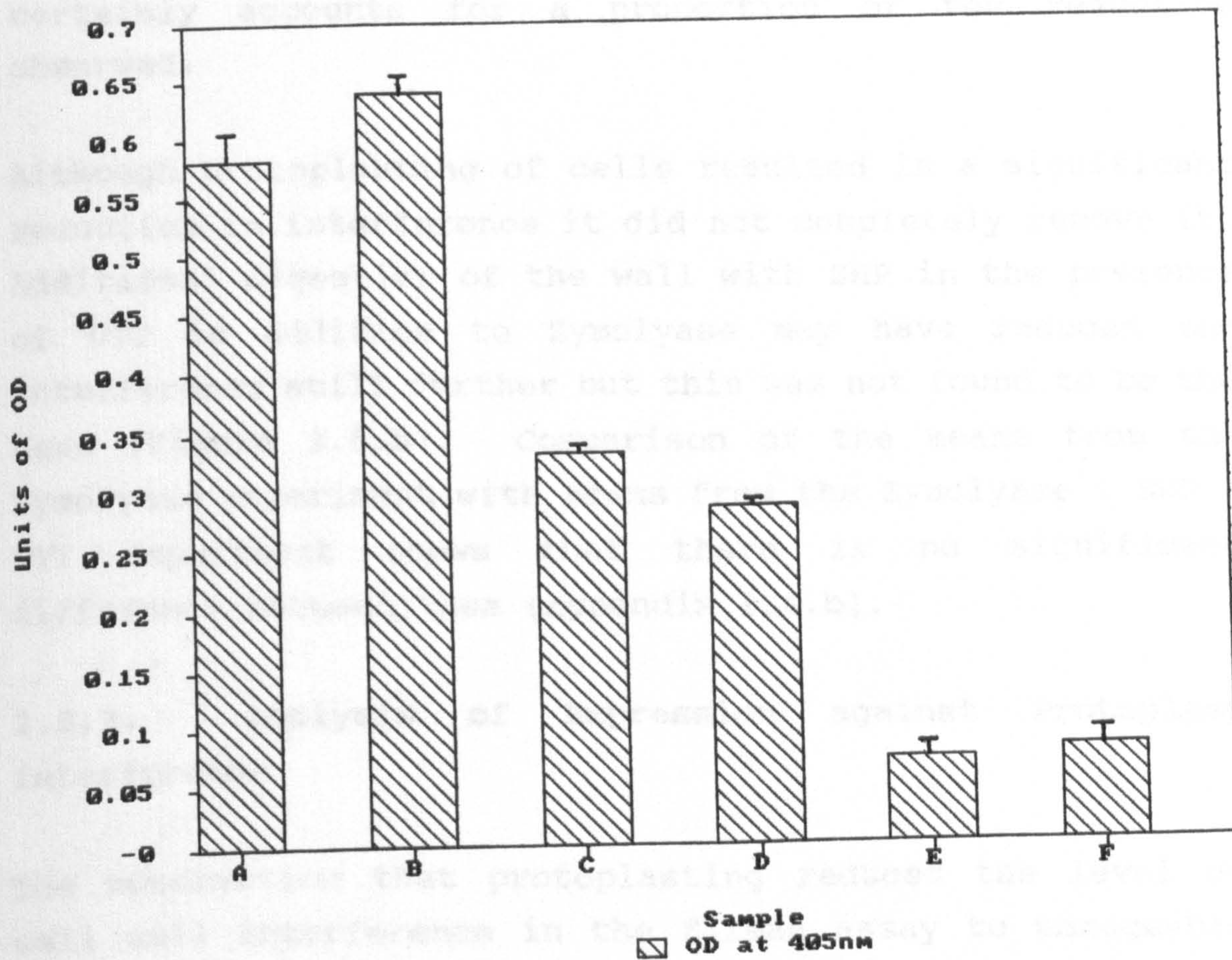




Figure 3.4. The effect of boiling the supernatant, use of a spun column, and absence of DTNB in the reaction to further investigate the cause of interference.

- A: Supernatant boiled for 5 min + Substrate
- B: Supernatant boiled for 5 min - Substrate
- C: Supernatant through Sephadex + Substrate
- D: Supernatant through Sephadex - Substrate
- E: Supernatant - DTNB + Substrate
- F: Supernatant - DTNB - Substrate







The number of washes of the protoplast pellet was found to have some effect: a further reduction of  $\approx 35\%$  in background for 8 washes as opposed to a single wash (Figure 3.5.a). Mean OD<sub>405nm</sub> of  $1.188 \pm 0.034$  and  $0.778 \pm 0.025$  ( $p = <0.001$ ) were obtained for 1 wash and 8 washes respectively. It should be noted that inevitably some loss of protoplasts occurs each time a wash is performed and this almost certainly accounts for a proportion of the reduction observed.

Although protoplasting of cells resulted in a significant reduction in interference it did not completely remove it. Additional digestion of the wall with SHP in the presence of DTT in addition to Zymolyase may have reduced the interference still further but this was not found to be the case (Figure 3.5.b). Comparison of the means from the Zymolyase experiment with means from the Zymolyase + SHP + DTT experiment shows that there is no significant difference between them (Appendix 1.4.b).

### 3.3.3. Analysis of Expression against Protoplast Interference

The observation that protoplasting reduced the level of cell wall interference in the Ellman assay to manageable proportions meant that protoplasts containing the pBM150-DACHE construct could be tested for expression of AChE. This was supported by a further observation that *Cx. molestus* AChE activity could be recorded against an imposed background of protoplasts. A rate could still be measured for a 1 in 8 dilution of mosquito homogenate (data not shown).

Significant rates of substrate hydrolysis were undetected from protoplasts containing pBM150-DACHE that had been



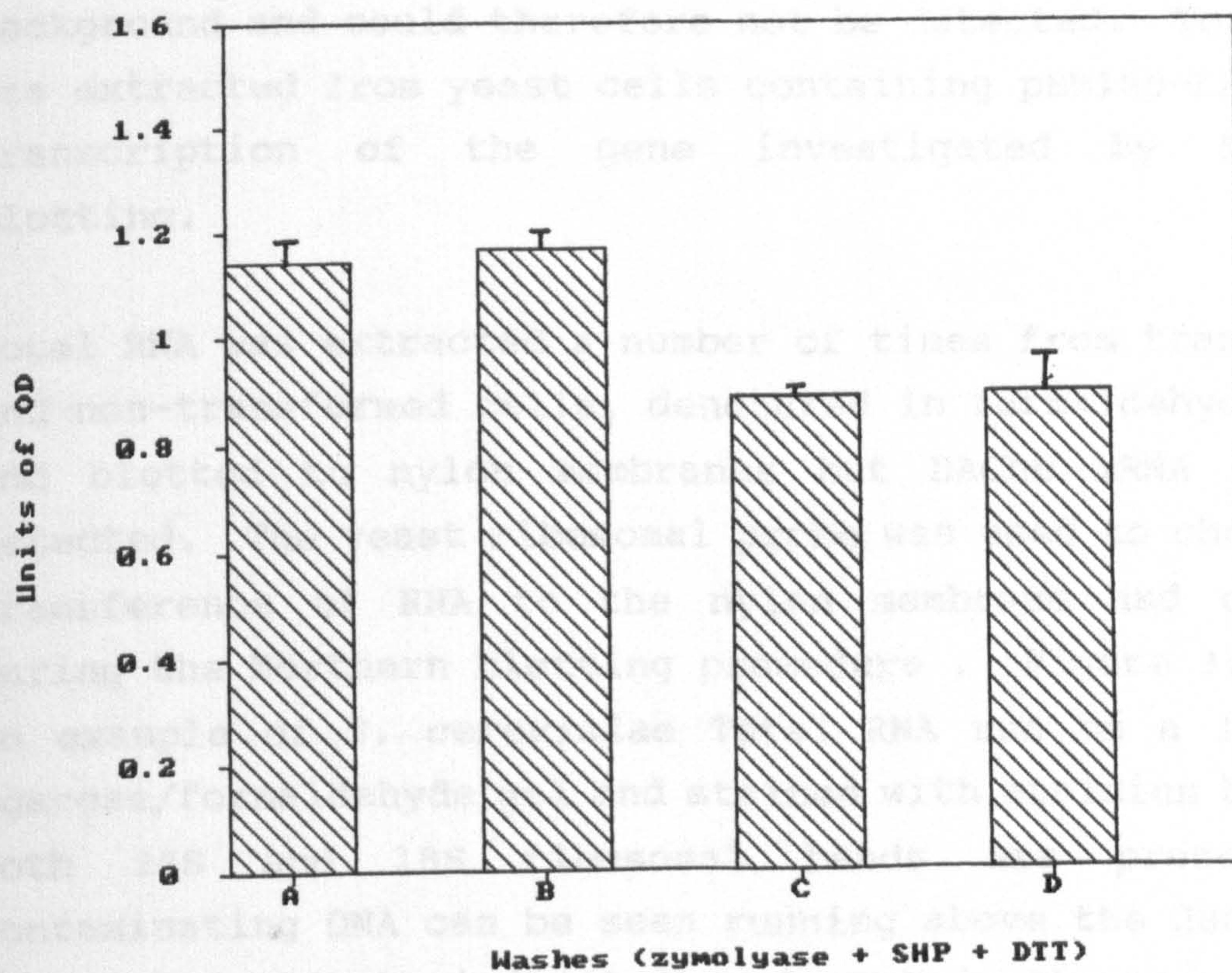
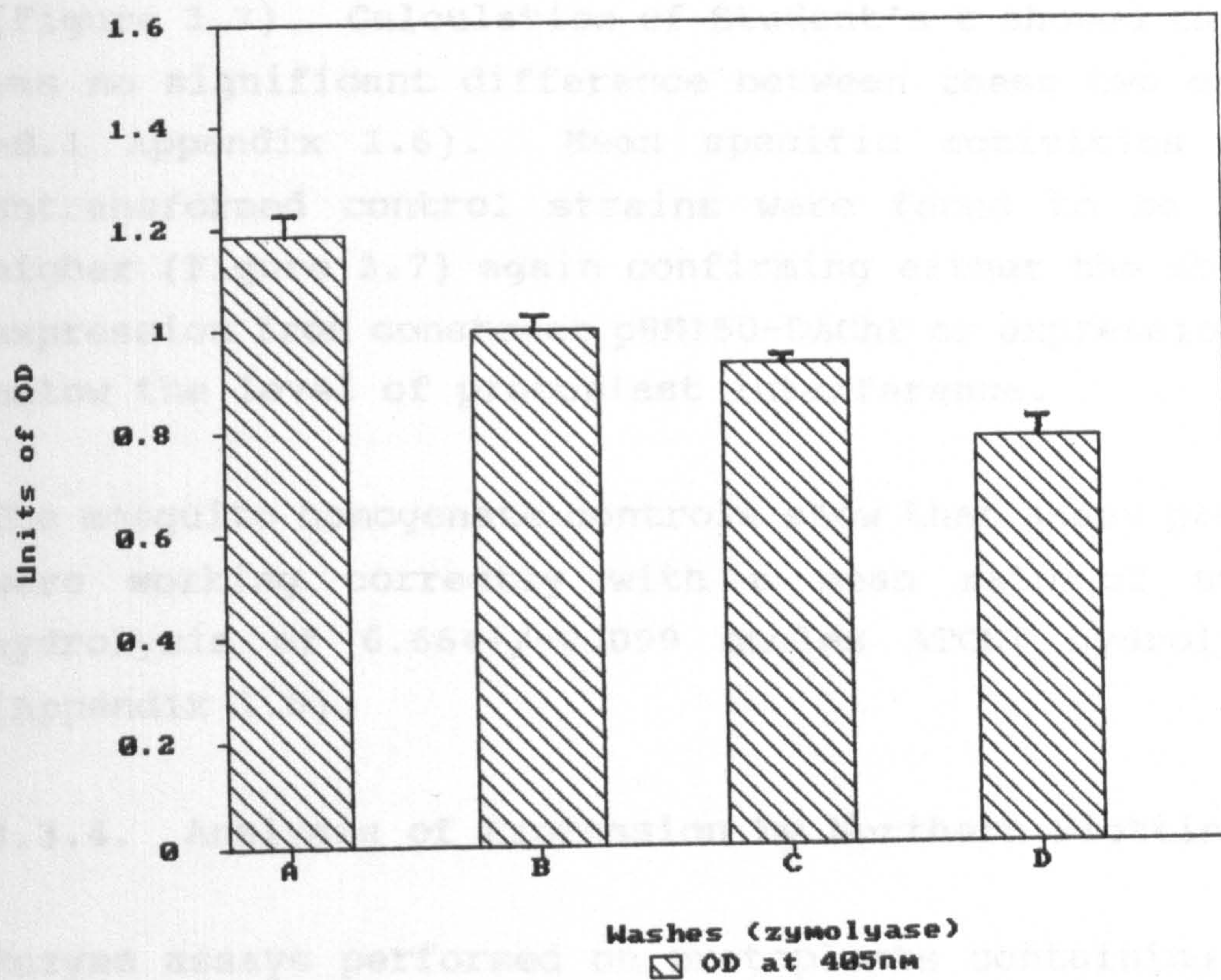
Figure 3.5.a. Levels of interference recorded following enzymatic removal of the cell wall with Zymolyase and subsequent washing of protoplasts.

- A: 1 Wash
- B: 2 Washes
- C: 4 Washes
- D: 8 Washes

Figure 3.5.b. Levels of interference recorded following enzymatic removal of the cell wall with Zymolyase together with additional digestion by SHP in the presence of DTT. As in Figure 3.4.b protoplasts were subsequently washed a varying number of times.

- A: 1 Wash
- B: 2 Washes
- C: 4 Washes
- D: 8 Washes







induced on galactose medium (Figure 3.6.). The mean specific activities obtained for induced and non-induced cells were  $12.15 \pm 0.653$  and  $11.81 \pm 0.756$  respectively (Figure 3.7). Calculation of Student's *t* showed that there was no significant difference between these two groups ( $p > 0.1$  Appendix 1.6). Mean specific activities for the untransformed control strains were found to be slightly higher (Figure 3.7) again confirming either the absence of expression from construct pBM150-DAC<sub>H</sub>E or expression levels below the level of protoplast interference.

The mosquito homogenate controls show that assay procedures were working correctly with a mean rate of substrate hydrolysis of  $6.664 \pm 0.099$  nmoles ATCHI hydrolysed/min (Appendix 1.5).

#### 3.3.4. Analysis of Expression by Northern Blotting

Enzyme assays performed on protoplasts containing pBM150-DAC<sub>H</sub>E indicated that either transformants were not expressing DAC<sub>H</sub>E or that expression levels were below background and could therefore not be detected. Total RNA was extracted from yeast cells containing pBM150-DAC<sub>H</sub>E and transcription of the gene investigated by Northern blotting.

Total RNA was extracted a number of times from transformed and non-transformed cells, denatured in formaldehyde gels, and blotted to nylon membranes but DAC<sub>H</sub>E mRNA was not detected. The yeast ribosomal probe was used to check that transference of RNA to the nylon membrane had occurred during the Northern blotting procedure. Figure 3.8.a. is an example of *S. cerevisiae* Total RNA run on a 1.1% w/v agarose/formaldehyde gel and stained with ethidium bromide. Both 28S and 18S ribosomal bands are present and contaminating DNA can be seen running above the 28S bands. The high pH of the initial phenol step in the procedure

Figure 3.6. Product formation above interference levels investigated for induced protoplasts containing pBM150-DAC<sub>H</sub>E assayed using a microtitre plate reader by the method of Ellman *et al* (1961).

Group	Well Co-ordinates
1) Cells transformed with pBM150-DAC <sub>H</sub> E induced on galactose	A1 - A3 B1 - B3 C1 - C3
2) Cells transformed with pBM150-DAC <sub>H</sub> E uninduced	D1 - D3 E1 - E3 F1 - F3
3) Untransformed cells 'induced' on galactose	A4 - A6 B4 - B6 C4 - C6
4) Untransformed cells 'uninduced'	D4 - D6 E4 - E6 F4 - F6
5) Mosquito homogenate control	A7 - A9 B7 - B9 C7 - C9
6) Blank	D7 - D9 E7 - E9 F7 - F9



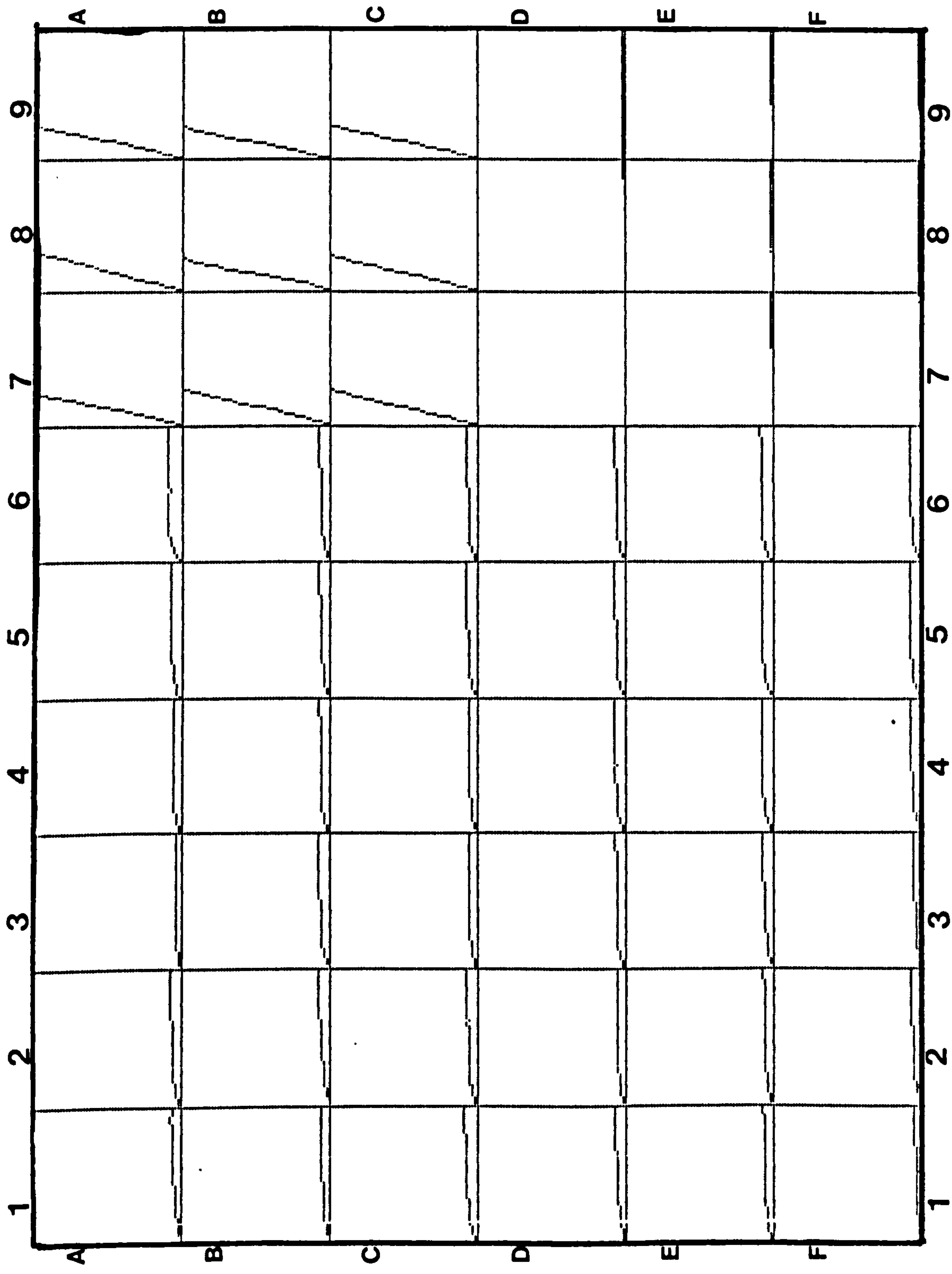


Figure 3.7. Transformed and untransformed cells assayed by the Ellman method compared in terms of specific activity.

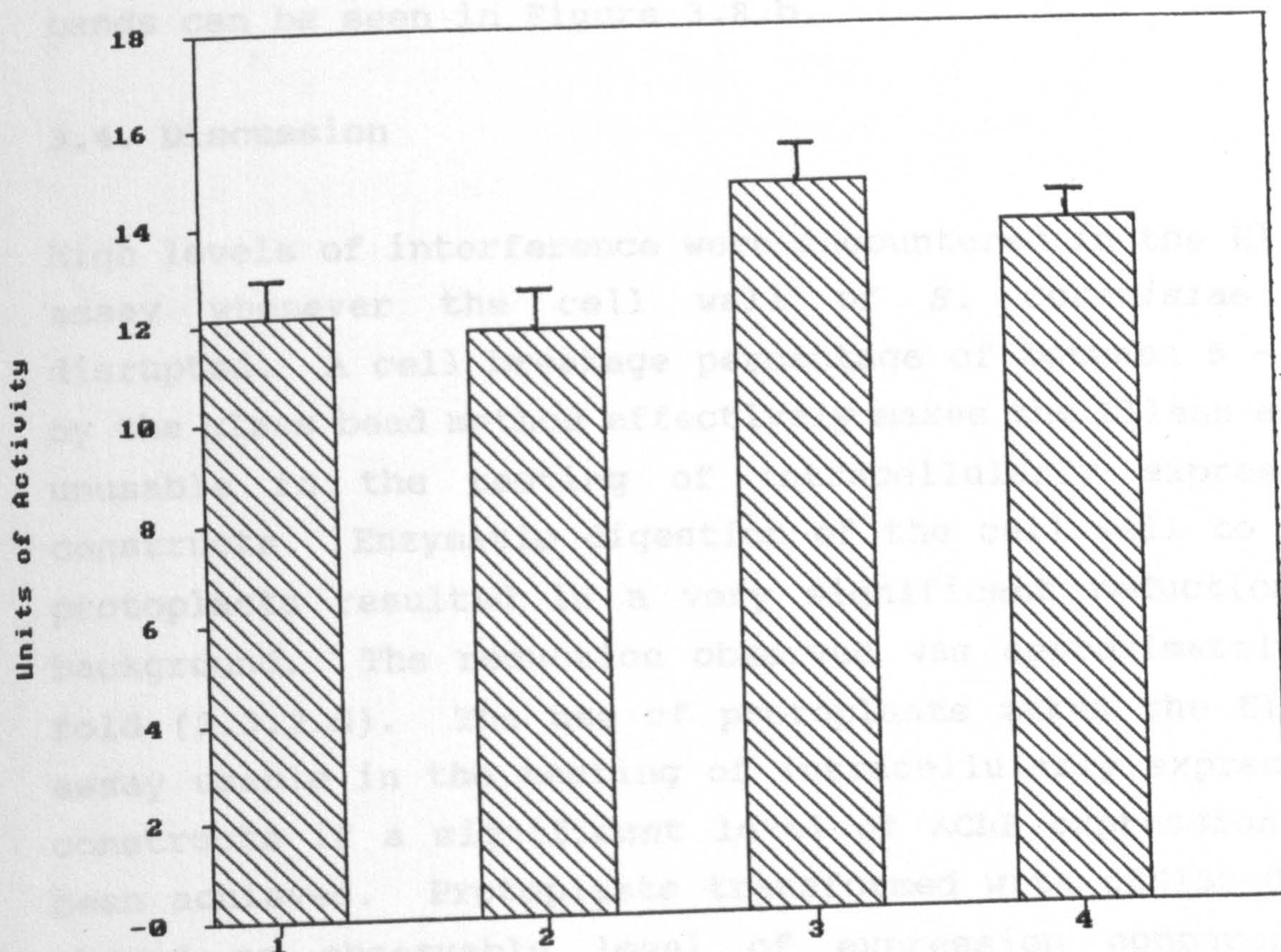
Group

- 1: Cells transformed with pBM150-DAC<sub>H</sub>E induced on galactose
- 2: Cells transformed with pBM150-DAC<sub>H</sub>E uninduced
- 3: Untransformed cells 'induced' on galactose
- 4: Untransformed cells 'uninduced'

Units of 'activity' refer to nmoles ATCHI hydrolysed/min/mg protein.



used causes DNA as well as RNA to be extracted to the aqueous phase. This should later be removed by a selective LiCl precipitation of RNA but this was found to be inefficient. Since RNA runs ahead of agarose gels the presence of contaminating DNA should not prevent the detection of an abundant message on a Northern blot. The Northern hybridization of the 6.1 Kb PstIII pVIA32 fragment probe to *S. cerevisiae* 28S and 18S ribosomal RNA can be seen in Figure 3.8.



showed no observable level of expression compared to control groups (figures 3.5 and 3.7). Since DASH was not expressed from construct pSH10-DASH or expressed at undetectable levels.

Investigation of transcription by Northern blotting indicated that transcription was either not occurring, occurring at very low levels, or that the transcript was unstable. The vector pSH10 does not contain any polyadenylation sites from *S. cerevisiae* derivation of the polylinker and this may have led to the production of long and unstable DASH transcripts. The possibility also exists that a mutation event significantly affected the



used causes DNA as well as RNA to be extracted to the aqueous phase. This should later be removed by a selective LiCl precipitation of RNA but this was found to be inefficient. Since RNA runs ahead on agarose gels the presence of contaminating DNA should not prevent the detection of an abundant message on a Northern blot. The Northern hybridization of the 6.1 Kb *Hind*III pY1rA12 fragment probe to *S. cerevisiae* 28S and 18S ribosomal RNA bands can be seen in Figure 3.8.b.

### 3.4. Discussion

High levels of interference were encountered in the Ellman assay whenever the cell wall of *S. cerevisiae* was disrupted. A cell breakage percentage of between 5 - 10% by the glass bead method effectively makes the Ellman assay unusable in the testing of intracellularly expressing constructs. Enzymatic digestion of the cell wall to make protoplasts resulted in a very significant reduction in background. The reduction observed was approximately 10 fold (3.3.2.d). The use of protoplasts makes the Ellman assay usable in the testing of intracellularly expressing constructs if a significant level of AChE expression has been achieved. Protoplasts transformed with pBM150-DACHE showed no observable level of expression compared to control groups (Figures 3.6 and 3.7). Either DACHE was not expressed from construct pBM150-DACHE or expressed at undetectable levels.

Investigation of transcription by Northern blotting indicated that transcription was either not occurring, occurring at very low levels, or that the transcript was unstable. The vector pBM150 does not contain any polyadenylation sites from *S. cerevisiae* downstream of the polylinker and this may have led to the production of long and unstable DACHE transcripts. The possibility also exists that a mutation event significantly affecting the

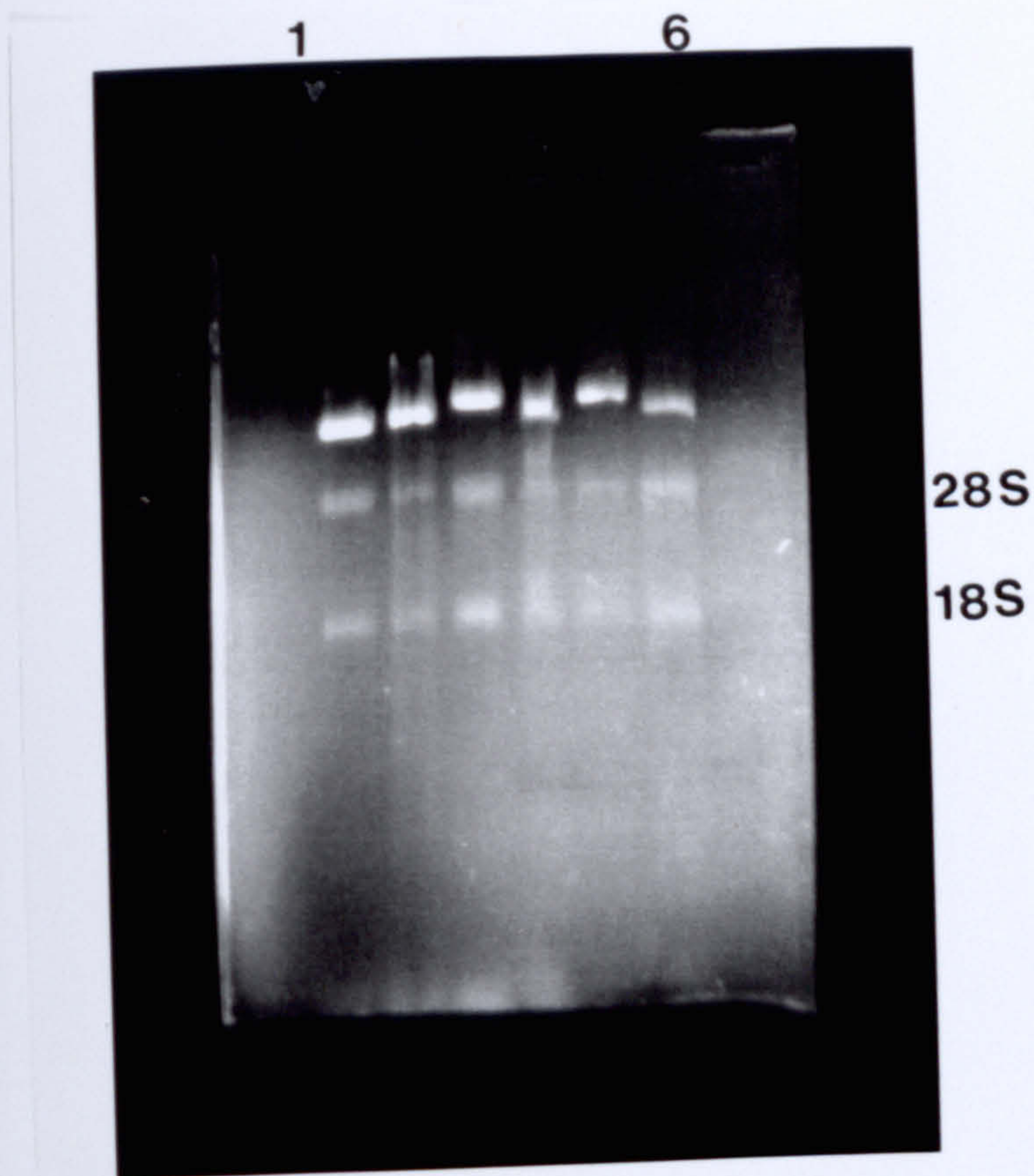


Figure 3.8.a. *S. cerevisiae* total RNA run in 1.1% w/v agarose containing 2.2M formaldehyde. 10 $\mu$ g RNA/lane.

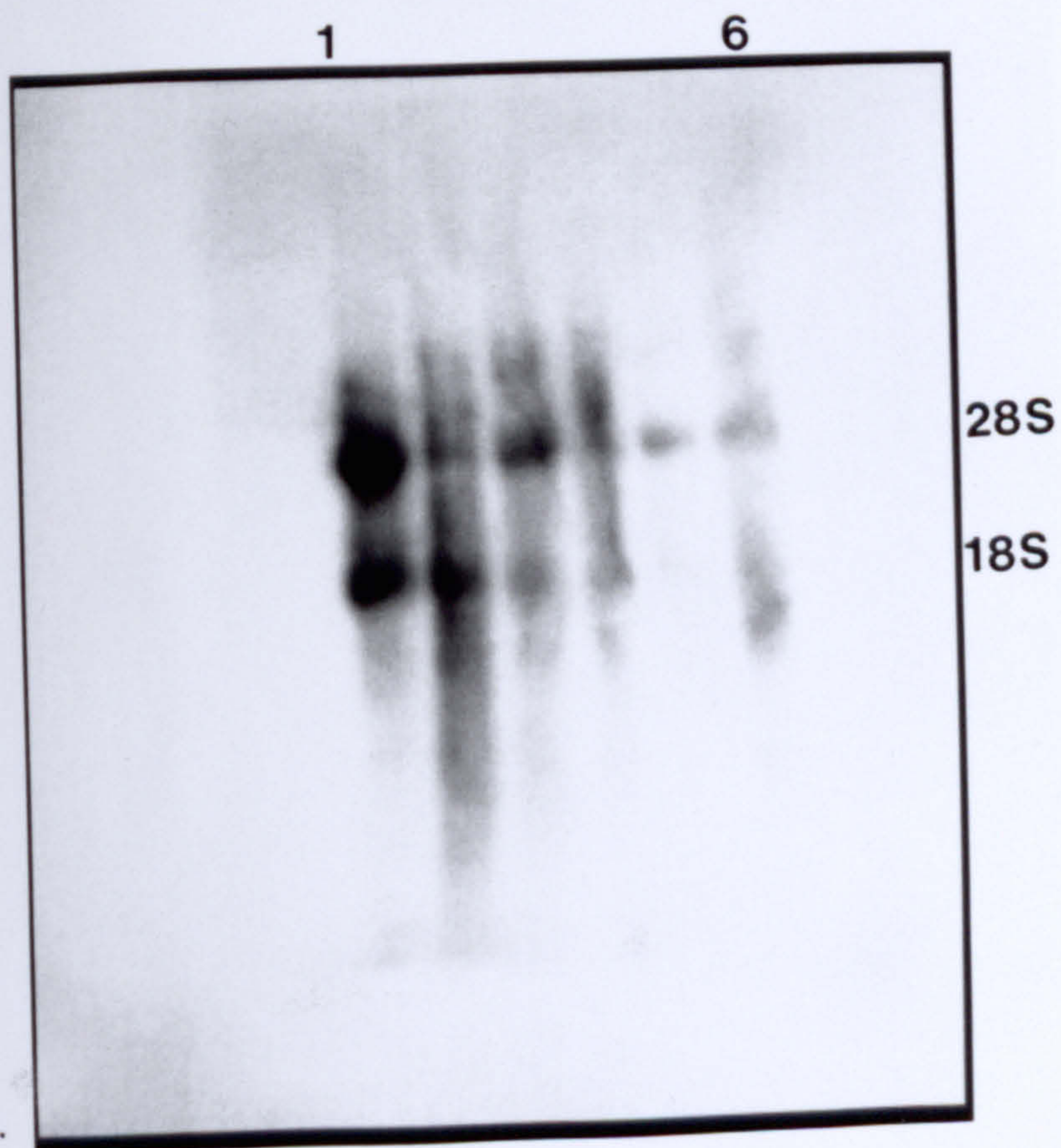
- Lane 1 - Transformed cells induced on galactose
- 2 - Transformed cells induced on galactose
- 3 - Transformed cells induced on galactose
- 4 - Transformed cells uninduced
- 5 - Non-transformed cells uninduced
- 6 - Non-transformed cells induced on galactose

Figure 3.8.b. Northern blot of gel in Figure 3.8.a. showing hybridization of 6.1 Kb *Hind*III pY1rA12 fragment probe (5 hr) to 28S and 18S *S. cerevisiae* ribosomal RNA bands.





A



B



*GAL10* promoter in the construct disrupted gene expression. Hybridizations were to total RNA and low transcript frequencies might be detected by hybridizing to poly A+ RNA. Such a low level of expression is not desirable for a heterologous system and the priority was to test other types of expression vector for a significant level of AChE expression.

The fact that DTNB was required in the formation of the interference suggests that a factor(s) interacts with the stain to produce the yellow anion 5-thio-2-nitrobenzoic acid on disruption of the cell wall. The results support the view that this is not due to any enzymatic action on the substrate ATCHI since its presence was not required for background to appear (Figure 3.2 and 3.3). Incubation with Proteinase K, which might have been expected to eliminate any endogenous enzymatic action, also had no effect (Figure 3.2).

The cell wall in *S. cerevisiae* consists of three major components: the polysaccharide glucan, mannoprotein (a glycoprotein with mannose side chains) and chitin (Ballou, 1976; 1982). These components form the matrix of the cell wall. Falcone and Nickerson as early as 1956 reported that the protein component of the cell wall contains relatively large quantities of sulphur, most of it in the disulphide -S-S- form. Numerous disulphide bridges are responsible for connecting the mannoprotein complexes that form the meshwork of the wall (Beran, 1968). It is these cross linkages that help determine the physical properties of the wall. Both -S-S- and -SH groups of the wall proteins are important for cell wall integrity and are also involved in the budding process (Beran, 1968). Disruption of the cell wall by mechanical shearing will result in exposed sulphur groups capable of reacting with the 5-dithiobis-2-nitrobenzoate ion of DTNB. It was found that these groups could not be successfully removed by centrifugation. This

could be due to the presence of a highly heterogenous population of exposed groups. Individual ions, in addition to exposed atoms still attached to cell wall proteins that are themselves a highly heterogenous mix of sizes from single liberated proteins to large particles of cell wall. Supernatants and cell pellets will therefore result in interference. A spun column will remove small ions but will not remove the groups still attached to proteins. A reduction in background by Sephadex G25 of the order of 40-50% was observed (Figure 3.4). Since interference levels of  $\approx 0.3$  OD<sub>405</sub> Units (Appendix 1.3) were recorded for 1 in 10 dilutions after spinning through Sephadex G-25 clearly gel purification is not a viable means of reducing the level of interference to manageable proportions.

The results show that protoplasting of cells prior to analysis is a way of utilizing the Ellman assay in the testing of *S. cerevisiae* transformed with expression constructs containing the DAcHE gene.



## CHAPTER FOUR SUBCLONING OF THE DACHE GENE

### 4.1. Introduction

Assays for the expression of AChE from the single copy shuttle vector pBM150 under the *GAL10* promoter indicated that active protein was either not being expressed or being expressed at such low levels as to be negligible.

Subcloning was therefore undertaken into three different yeast expression vectors to maximise the possibility of successful expression in *S. cerevisiae*. Two of the vectors used were high copy number plasmids containing the yeast 2 $\mu$ m origin of replication. p2UG is a hormone inducible vector which utilizes the yeast *CYC1* promoter for the expression of heterologous genes and pG3 a constitutive vector containing the powerful *GPD* promoter (1.5.2). The third vector used was a secretion vector pPIC9 developed by Invitrogen for use with *P. pastoris* and used with some success for the expression of two vertebrate AChEs (Morel and Massoulie, 1997) (1.5.7.b). Successful secretion of the insect AChE to the culture medium would have the benefit of completely eliminating cell wall interference in the Ellman assay (3.3.1) and the advantage that supernatants from mutant cultures could be tested directly. Secretion of the enzyme would also simplify purification procedures if required for further characterization or production. It was also interesting to examine the differences that may exist in the expression of vertebrate and invertebrate AChEs in *P. pastoris*.

Difficulties were encountered in the subcloning of the DACHE gene. The limited number of restriction site options available within the polylinkers of the vectors and the length of the gene (>2Kb) partially accounted for this. Restriction sites in common meant that methylation of these

sites within the gene was required for most possible strategies leading to losses of DNA and problems with suitable quantities available for use in final ligation steps. The lack of a simple screening system, such as is available with *E. coli* vectors, was also a rate determining step. Phosphatasing of the vector arms of p2UG and pG3 led to problems of gene ligation. Subcloning into unphosphatased vectors required the use of a radiolabelled screening system. Sites in common made the use of two restriction enzymes ensuring correct orientation extremely difficult and therefore positive clones isolated contained the gene in either orientation. A positive clone containing the gene in the correct orientation in vector pG3 was finally isolated from the replating and rescreening of  $\approx 500$  colonies.

The subcloning strategy adopted for the secretion vector had to ensure that the  $\alpha$ -factor mating signal was fused in frame with the coding sequence of the DAcHE gene which contained a small section of leader sequence (71 nucleotides) upstream of the start codon. A relatively complex strategy was again required which included the restriction digest and subsequent recovery by ultracentrifugation of a specially designed double stranded oligonucleotide.

#### 4.2. Materials and Methods

Details of shuttle vectors are given in 2.4. Details of subcloning manipulations are given in 2.5, 2.6, 2.8, 2.11 - 2.16, 2.20, 2.21. The DAcHE cDNA sequence with the important structural features highlighted is shown in Figure 4.1. A detailed restriction map of the DAcHE cDNA fragment including all of the salient restriction sites and indicating the location of the coding region relative to restriction sites is given in Figure 4.2.



Figure 4.1. The cDNA sequence of the DAcHE gene (after Fournier et al, 1989).

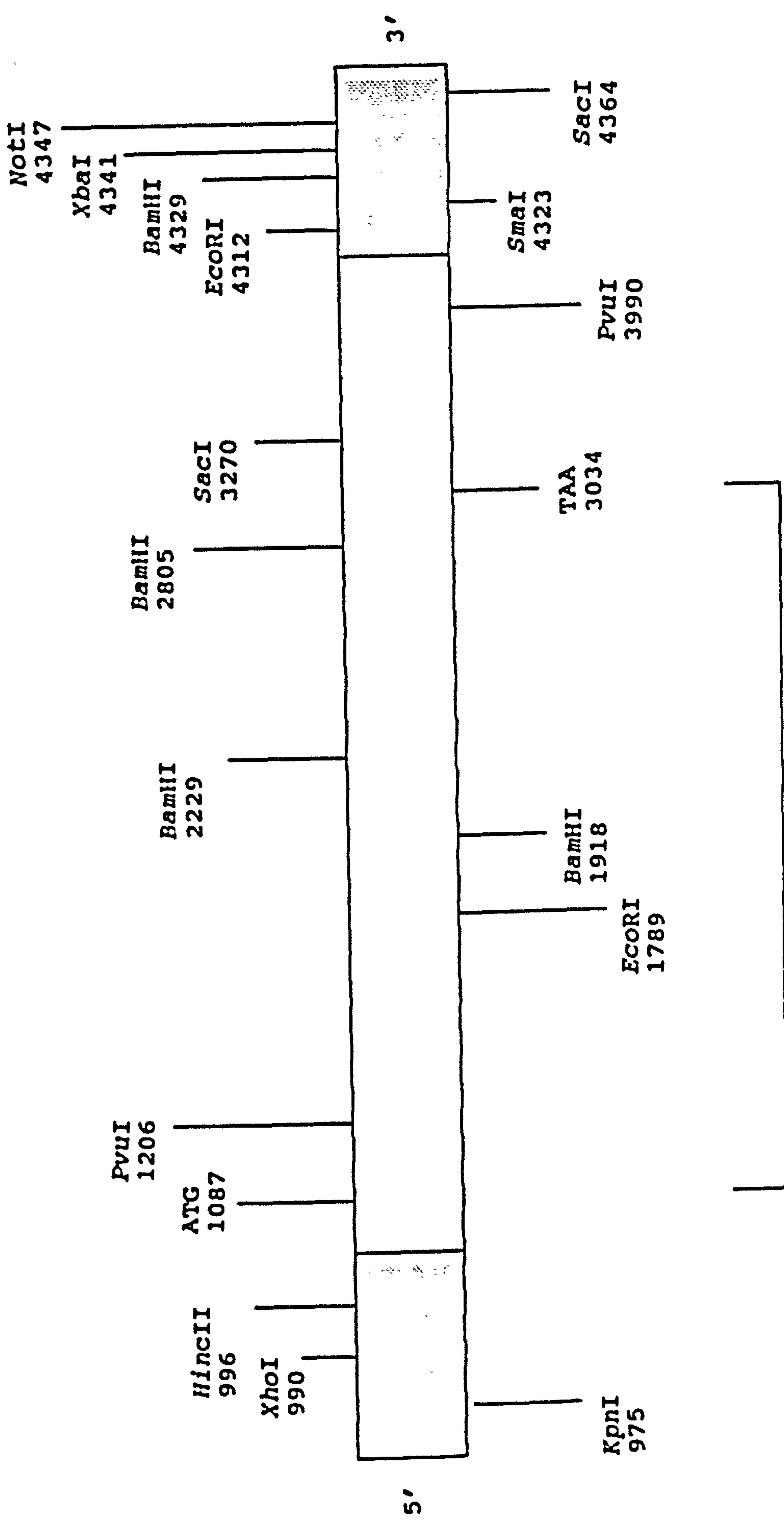
mRNA start site (position 1). A putative TATA box resides 40bp upstream in the sequence GAGAGAGAGA TGGTATAATA GTCGCTCAAA AGCTGTCGAG AGAGAGGGAG AGAAAAGAGA G... mRNA splice sites are after positions 562, 1409, 1548, 2094, 2302, 2445, 2595, 2759, and 2923. The two potential polyadenylation sites are underlined at positions 3981 and 4262. START and STOP codons of the open reading frame are double underlined at positions 1087 and 3034. The start of the DAcHE cDNA sequence in Bluescript KS+ is underlined at position 1015. The leader sequence upstream of this position was deleted prior to subcloning procedures. Multiple translation start starts are indicated at cDNA positions 349, 355, 433, 522, 798, 879. The start of the mature protein at position 1201 (Val<sup>39</sup>) is underlined and thus the preceding sequence codes for the 38 amino acids of the signal peptide. The consensus sequence surrounding the active site Ser<sup>276</sup> is underlined from position 1900 - 1930. The other members of the catalytic triad Glu<sup>405</sup> and His<sup>518</sup> are underlined at positions 2299 and 2638. The hydrophilic sequence from position 1504 - 1627 (the site of cleavage of the 75 KDa precursor) is shown in brackets. The hydrophilic sequence contains two potential sites of tryptic cleavage at Arg<sup>148</sup> and Lys<sup>159</sup> (both underlined). The hydrophobic sequence from 1628 - 1776 immediately following is also shown in brackets. The hydrophobic portion at the carboxy-terminal end of the protein is indicated by square brackets. Potential sites of Asn-linked glycosylation at amino acid positions 126 (1462 cDNA), 174 (1606), 331 (2077), 531 (2677), and 569 (2791) are shown as a dotted underline. The eight cysteine residues of the mature protein are also shown as a dotted underline at amino acid positions 104 (1396), 130 (1477), 328 (2067), 330 (2074), 345 (2119), 480 (2524), 598 (2878), and 615 (2929).



+	GAGTGAAGC	ATAGTCCCGC	TATTTTGCCG	AGAGAAATAA	AGAGCAGAAA	AGCAATGTCC	60
	GTTTAAAGTA	CGCATTGCAG	TGCCTTTTAG	CTTCAGTTTA	GCTAGAGACA	TCGTCTGTGT	120
	TTGAACGGCT	GACACATTTT	CGAGTTCAGC	GGACTAGCTG	GACTACGCCA	CACTAGTATT	180
	CAGTTCAGTG	CGCAAAAAAG	GCCAAAAAGA	AAACCAGAAA	AACTTTTAAA	ATAGCCAGGA	240
	TAAAAGAAGG	CAGACCGTAC	ATCTTTTAAAG	GGAAACACCA	TACCCCTTCT	GTCTCAAGC	300
	CAGCTCTTTC	AAACAAACAC	ACACATACAC	ATACCTGGCA	TGCATATGGC	ATAACACGGC	360
	CAGAAAAGGA	TATTCATTAG	TCAGATTCTG	TGTGTG/TAA	AGTGAAGTTT	TGGTGCTAAA	420
	ATCTAGTATT	CAATGCAAGC	AGCCCTGCAC	TTTATTTTACA	GCTTGGAAAA	TTGGCAAATC	480
	AGAGAGTCGG	AGAGTTTTTA	ACATCAAACA	TAAACAGAGT	CATGTAGTGA	GCGACCGAGG	540
	ACAACAGTTT	CAGCGCACAA	AGAGTTCAAA	CACATCCGCA	GACGGAGAAG	AAAGGCCAAG	600
	CAGACGGAGT	GGGCTCTGCA	AAAGTGCAAC	AGCTGCTCTG	AAAACCGAAA	CGAATAGAGG	660
	ACGCCAACTG	AAAACCATTT	CAAACCTCTAA	GCCAAGTCCA	ATAAAGCCGA	GACCCGATTC	720
	GAGTGGCGAG	TACCGAGCAA	CCAGTACCCA	GTTTCCAGTT	CCCAGATTCC	GGATTCCAAA	780
	GCTGCTGCGG	CGGGAGAATG	TGTAGTGCAA	CCCACGGGCG	GCACGTTCAA	GTGGCAATTG	840
	TGGCAGCAGA	GGCGAACTCA	ACCGGATAAT	AAGTTGTGGA	TGCGGCTTGT	AGCGCCGGGC	900
	AGGCATCCCG	AATCCGCCAG	ACACACCACT	TCCGTACCGA	GTTACCCAGC	GAGCAGAAAT	960
	CGCGAATCCG	CAACAGCCAG	AGAACCAGAG	ACAAGTGGTC	AGTGCCAGTG	ACGTCAGGCC	1020
	AAAGTAGGCC	ACCACCACCC	ATCGCTACCG	CTTTAGCCAG	CCAACGACGC	CCTGCGCATC	1080
	CGCGTCATGG	CCATCTCCTG	TCSGCAGAGC	AGAGTCCTGC	CCATGTCTTT	GCCCCTGCTT	1140
	CTGACCATCC	CGCTGCCCTT	GGTGCTGGTG	CTATCACTGC	ACCTGTCCGG	CGTCTCGGGC	1200
	GTCATCGATC	GCCTGGTCTG	GCAGACATCC	TCCGGACCTG	TACGCGGTCT	CTCCGTGACG	1260
	GTGCAGGGCA	GGGAGGTGCA	TGTCTACACG	GGCATCCCCT	ACGCCAAGCC	GCCCGTCGAG	1320
	GACCTGCGCT	TCCGAAAGCC	GGTTCCCGCG	GAGCCATGGC	ACGGCGTCTT	CGACGCCACC	1380
	GGGTTATCCG	CCACCTGCGT	CCAAGAGCGT	TACGAGTACT	TCCCCGGCTT	CTCCGGCGAG	1440
	GAGATCTGGA	ACCCCAACAC	CAACGTGTCC	GAGGACTGCC	TCTACATAAA	TGTCTGGGCG	1500
	CCGCAAAAGG	CCCGACTTCG	CCATGGGCGG	GGTGCCAAAG	GGGGTGAGCA	CCCCAATGCC	1560
	AAACAGGGCG	ACACTGACCA	TCTCATCCAC	AACGGAAATC	CGCAGAACAC	GACCAACGGA	1620
	CTGCCGATTC	TGATCTGGAT	CTATGGCGGT	GGCTTCATGA	CCGGATCGGC	CACCCCTGGAC	1680
	ATCTACAATG	CGGATATCAT	GGCGCGCGTG	GGCAATGTAA	TAGTGGCCTC	CTTCCAGTAT	1740
	CGGGTGGGAG	CCTTTGGGTT	CCTGCACCTG	GCGCCGAAAA	TGCCGTCCGA	ATTCCGGGAA	1800
	GAGGCGCCCG	GCAATGTGGG	CCTATGGGAT	CAGGCACCTG	CCATTGCTTG	GCTGAAGGAC	1860
	AACGCTCATG	CCTTCGGCGG	AAATCCGGAG	TGGATGACAC	TGTTCTGAGA	GTCCGCTGGA	1920
	TCCAGTTCGG	TGAATGCCCA	GCTCATGTCC	CCGGTGACGA	GGGGTCTGGT	CAAGCGCGGA	1980
	ATGATGCAGT	CGGGCACTAT	GAACGCCCCC	TGGAGCCACA	TGACCTCCGA	GAAGGCCCTG	2040
	GAGATCGGCA	AGGCGCTGAT	CAACGACTGC	AACCTGCAATG	CATCTATGCT	GAAGACCAAT	2100
	CCCGCTCACG	TGATGAGCTG	CATGCGTTCC	GTGGACGACA	AGACCATATC	GGTGCAGCAG	2160
	TGGAACCTCT	ACTCGGGCAT	CCTCAGCTTT	CCCTCGGGCG	CCACCATTGA	TGGTGCGTTC	2220
	CTGCCGGCGG	ATCCCATGAC	GCTGATGAAG	ACGGCGGATC	TGAAGGACTA	CGACATCCTG	2280
	ATGGGAAATG	TCAGGGATGA	GGGCACTTAC	TTCTTGCTGT	ACGATTTCAT	CGATTACTTC	2340
	GATAAGGACG	ATGCCACGGC	CCTGCCACGG	GACAAATACC	TGGAAATTAT	GAACAATATT	2400
	TTTGGAAGG	CAACGCAAGC	GGAACGCGAG	GCCATCATTT	TCCAGTATAC	CAGTTGGGAA	2460
	GGCAATCCTG	GCTATCAGAA	CCAGCAGCAA	ATCGGACGCG	CCGTGGGCGA	TCACTTCTTC	2520
	ACCTGCCCCA	CCAACGAGTA	TGCCCAGGCT	CTGGCGGAGC	CAGGCGCTTC	CGTGCACAC	2580
	TACTACTTTA	CACACCGCAC	AAGCACCTCA	TTGTGGGGCG	AATGGATGGG	CGTGCTGCAC	2640
	GGCGATGAGA	TGGAATACTT	CTTTGGCCAG	CCGCTGAACA	ACTCCCTGCA	GTATCGACCT	2700
	GTGGAGCGTG	AGCTGGGCAA	GCGTATGCTC	AGTGCGGTCA	TCCAGTTTGC	TAAGACGGGA	2760
	AATCCCCTCT	AGGATGGCGA	GGAGTGGCCC	AACCTTCTCCA	AGGAGGATCC	CGTCTACTAT	2820
	ATTTTCAGCA	CCGACGATAA	GATCGAGAAA	TTGGCCAGGG	GTCCCTTTGG	GGCTCGCTGC	2880
	TCGTTCTGGA	ATGATTACTT	GCCAAAAGTC	AGGAGTTGGG	CAGGTACTTG	CGATGGCGAT	2940
	TCSGGAAGTG	CTTCCATATC	CCCGAGGCTC	CAGCTCCTTG	GAATCGCTGC	TCTGATCTAC	3000
	ATCTGCGCCG	CATTGCGAAC	CAAAAGGGTT	TTCTAAAAGA	AGACCACTCC	CGGAAAATAC	3060
	GACATATACA	CACAAACTGG	AACACACACT	CACACACCCC	ATCACAACCG	CAACCATACA	3120
	CACTTAAAGT	TAGAACACGT	ATATTAATAA	AACCAAATTT	TTAGTAGGCG	CAGACATTTG	3180
	TGTAGGAGCA	GGTGATTTGT	TTACAGAACT	TAGTAGGATC	GGTTTGAGCA	ATGCGATGGT	3240
	AATTGCTGAA	CATTTGTAAA	TTGAAATTAG	AGCTCTACGC	ACCCTTAGGC	AGTCAGATGG	3300
	TCAGGCAGGG	TCCTTCGAAG	GATTCCTGGAC	ATCCTGGCCA	GACACCAGAG	ACCACTGTAG	3360
	CCACATGCCA	CACGCCACGG	CCCATAGGCC	AGGCCCAAAC	CAAATTGTTT	TTTGTGAAGC	3420
	ACAATGCTAA	ATCGCTTAAT	TTGAAAAATT	GGCCAAGGGA	TGGGCGGAAT	TTTTGATAAA	3480
	TATTAATGCA	GATAGAACTA	AAATGTTTGT	CATTTTGCCT	TTGCGGCAGC	CAAAAAGAAA	3540
	CAAAGTTTAC	AGATACTCGT	TTACCGAACG	GAAAACCTCC	TCAGATGACA	GACACTTTAT	3600
	TGCCATGCCC	ACATTCTTGA	GCGATATTTG	AGCGCATTTA	ATGGCACAAAT	TTAAGCATTC	3660
	AGTTCAAACC	GGAAAAAAGT	CCTTGTTGCC	TGAAGATAGT	TGGCCAAATT	GCATTGGTTT	3720
	CATGCTTAGG	TTGAGGGTTC	GTTTGATTTT	GATTACACAT	CCCAAGGAAA	AACTATACTC	3780
	GAAAAATTGA	ACCACTCGAT	TCTTATGAGA	AACCCCAAAT	GGACATTTTC	GGAAAAATCA	3840
	CAATTGTATT	GTACAAAAAT	TGCCATTGGT	TTATTAGAAG	AAAACATAAA	GTAAAAACCA	3900
	AAAAAATAAA	CGCGGACTAA	TAGCCCCAAG	CCAAGCACTA	CCCAAGACAA	ATTTTTTTCG	3960
	AACAAATTTA	ACAAATTGTA	ATAAAAGAATC	GATCGAAAAT	GTGTTGTAAG	CGTAGTACTG	4020
	GGCAAAGACG	AAGATGATAT	TTATAATGCA	AACATATCAG	TATATTATTG	TATTTGTACT	4080
	AAACGTTTTA	CAAATTGGCT	TAGTGAGAAA	ATTGGACGGA	GGAAGATGAA	AGATGAAATG	4140
	ATTTAATACT	AAATAATGGT	AATTATAAAA	TTTATACACA	TAGCGAAATT	GGATCAATGA	4200
	TTAACACAAA	ACGTATTGTA	TGATGAGTAA	CGTATATGTA	CTTGGTATGT	CTATATCTAT	4260
	AAATAAATAT	TGTTCAAAAC	GTAATCCAA	CAAAAAAATA	AAAAAATA..	.....	4308



**Figure 4.2. Restriction map of the DACH<sub>E</sub> cDNA fragment used in subcloning procedures. The salient restriction sites are marked and cDNA position is numbered as in Figure 4.1. Flanking sections of the Bluescript KS<sup>+</sup> polylinker are shaded and the location of the coding region relative to individual restriction sites is shown.**





#### 4.2.1. Subcloning into Plasmid p2UG

The strategy adopted for subcloning the *D. melanogaster* AChE gene into the *Bam*HI site in the polylinker of p2UG is illustrated in Figure 4.3. The gene was excised on a *Sma*I-*Hinc*II fragment and gel purified to give a linear gene fragment with blunt ends. The vector was linearized with *Bam*HI and gel purified. To minimize self-ligation 5' phosphates were removed from the ends of the vector using Calf Intestinal Alkaline Phosphatase (CIAP). The linear *Bam*HI phosphatased p2UG was then ligated to the methylated DACHÉ *Sma*I-*Hinc*II gene fragment with *Bam*HI digested linkers and transformed into *E. coli*. Minipreps were performed on colonies from experimental plates to check for the presence of inserts. Ligations of the *Bam*HI digested insert into unphosphatased p2UG were transformed into *E. coli*, colony lifts performed, and probed using a 1.5 Kb *Eco*RI-*Sac*I segment of the DACHÉ cDNA (Figure 4.7.a.) and an Amersham enhanced chemiluminescence (ECL) kit in accordance with the manufacturer's instructions.

Table 4.1. Ratios of vector:insert used in subcloning into phosphatased p2UG.

Plate	Vector (ng)	Insert (ng)	Units of T4 DNA Ligase (New England Biolabs)
1	≈200	≈80	4
2	≈200	≈80	40
3	≈300	≈80	4
4	≈200	0	4
5	≈200 <sup>UP</sup>	0	4
6	0	0	0

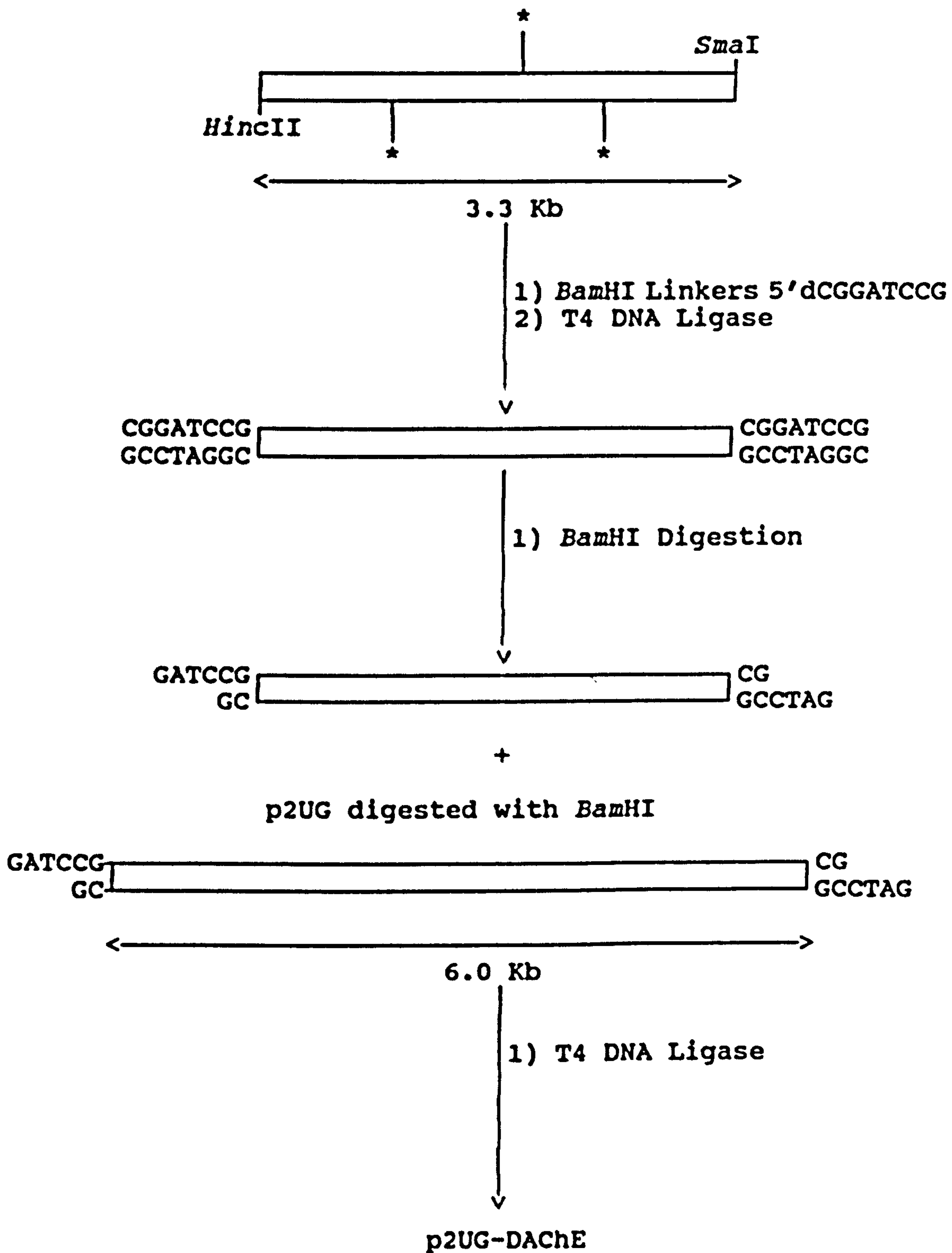
UP - Unphosphatased vector control ligation.

**Figure 4.3. The strategy adopted for subcloning into plasmid p2UG involved methylation of the three endogenous BamHI sites within the DACE cDNA followed by blunt end ligation of BamHI linkers.**



## Strategy for Subcloning into Plasmid p2UG

### *D. melanogaster* AChE cDNA



\* Methylated *Bam*HI sites

Table 4.2. Ratios of vector:insert used in subcloning into unphosphatased p2UG.

Plate	Vector (ng)	Insert (ng)	Units of T4 DNA Ligase (New England Biolabs)
1	≈200	≈100	4
2	≈200	≈100	4
3	≈200	≈100	4
4	≈200	0	4
5	0	0	0

#### 4.2.2. Subcloning into Plasmid pG3

The strategy adopted for subcloning the DACH<sub>E</sub> gene into the *Sac*I site in the polylinker of pG3 is illustrated in Figure 4.4. A single *Sac*I site in plasmid pG3 was created by excision of the 1.7 Kb 'stuffer' fragment. pKS-DACH<sub>E</sub> was first linearized with *Hinc*II and gel purified. *Sac*I linkers were ligated to this, digested with *Sac*I and *Kpn*I and the 2.3 Kb *Sac*I gene fragment gel purified. In one set of ligations pG3 was linearized by *Sac*I digestion, phosphatased with CIAP, ligated together with the 2.3 Kb *Sac*I fragment of the DACH<sub>E</sub> cDNA and transformed into *E. coli* (Table 4.3). Minipreps were performed on transformants arising from vector/gene ligations to see whether any contained an insert. In another set of ligations pG3 was linearized by *Sac*I digestion, ligated directly with the 2.3 Kb gene fragment, transformed into *E. coli* (Table 4.4.) and probed using a <sup>32</sup>P labelled gel purified 1.5 Kb *Eco*RI-*Sac*I fragment of the DACH<sub>E</sub> cDNA. Cloning of the gene fragment with ligated *Sac*I linkers into pUC19 (Gibco BRL) enabled recombinants to be obtained through blue/white screening (2.17). It was then possible



Figure 4.4. The strategy for subcloning into plasmid pG3 involved the blunt ligation of *SacI* linkers to a *HincII* linear gene-vector followed by *SacI* digestion. A single *SacI* cloning site was created in pG3 prior to ligation of the gene fragment.

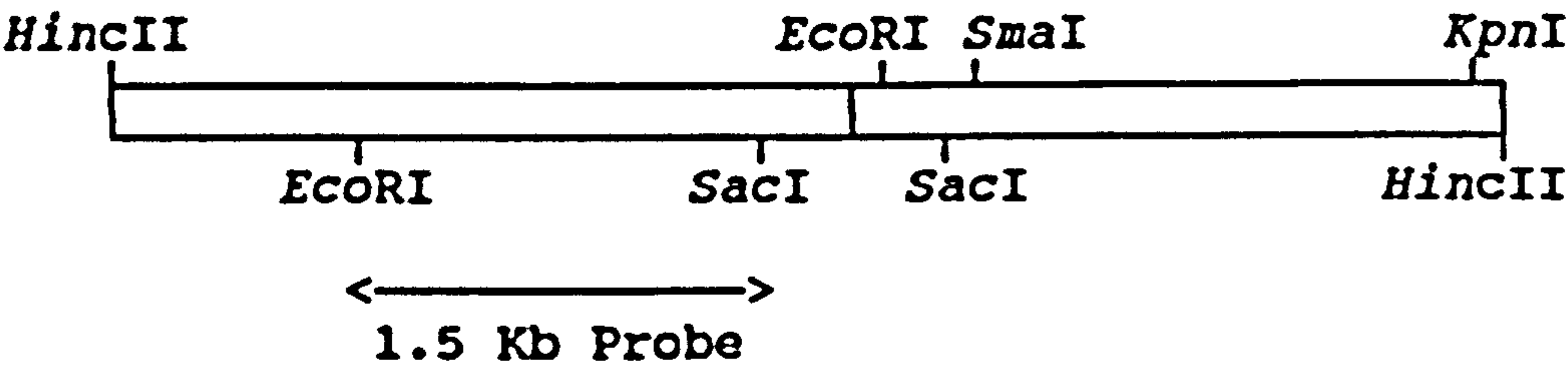
\* Incompatible *KpnI* end.

# Strategy for Subcloning into Plasmid pG3

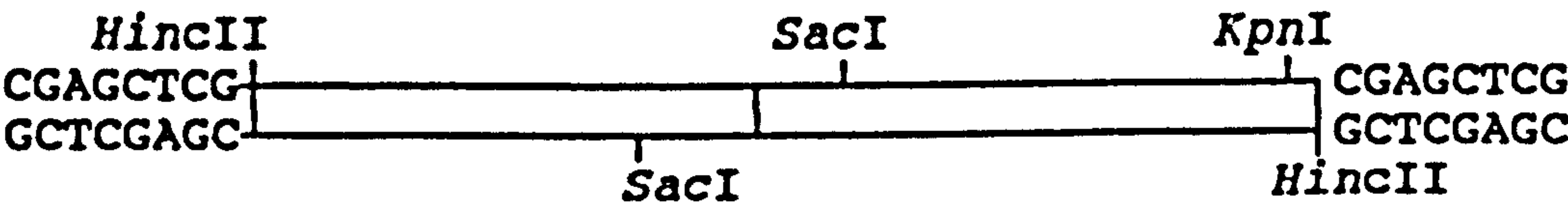
## Preparation of Gene

*D. melanogaster* AChE cDNA

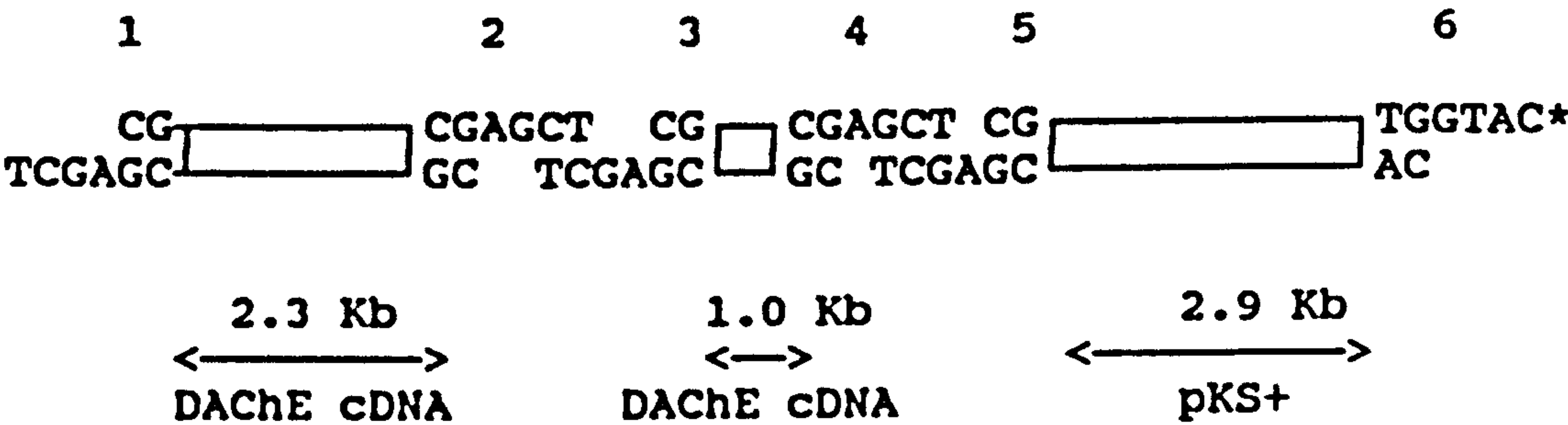
pKS+



- 1) SacI Linkers d5'CGAGCTCG
- 2) T4 DNA Ligase



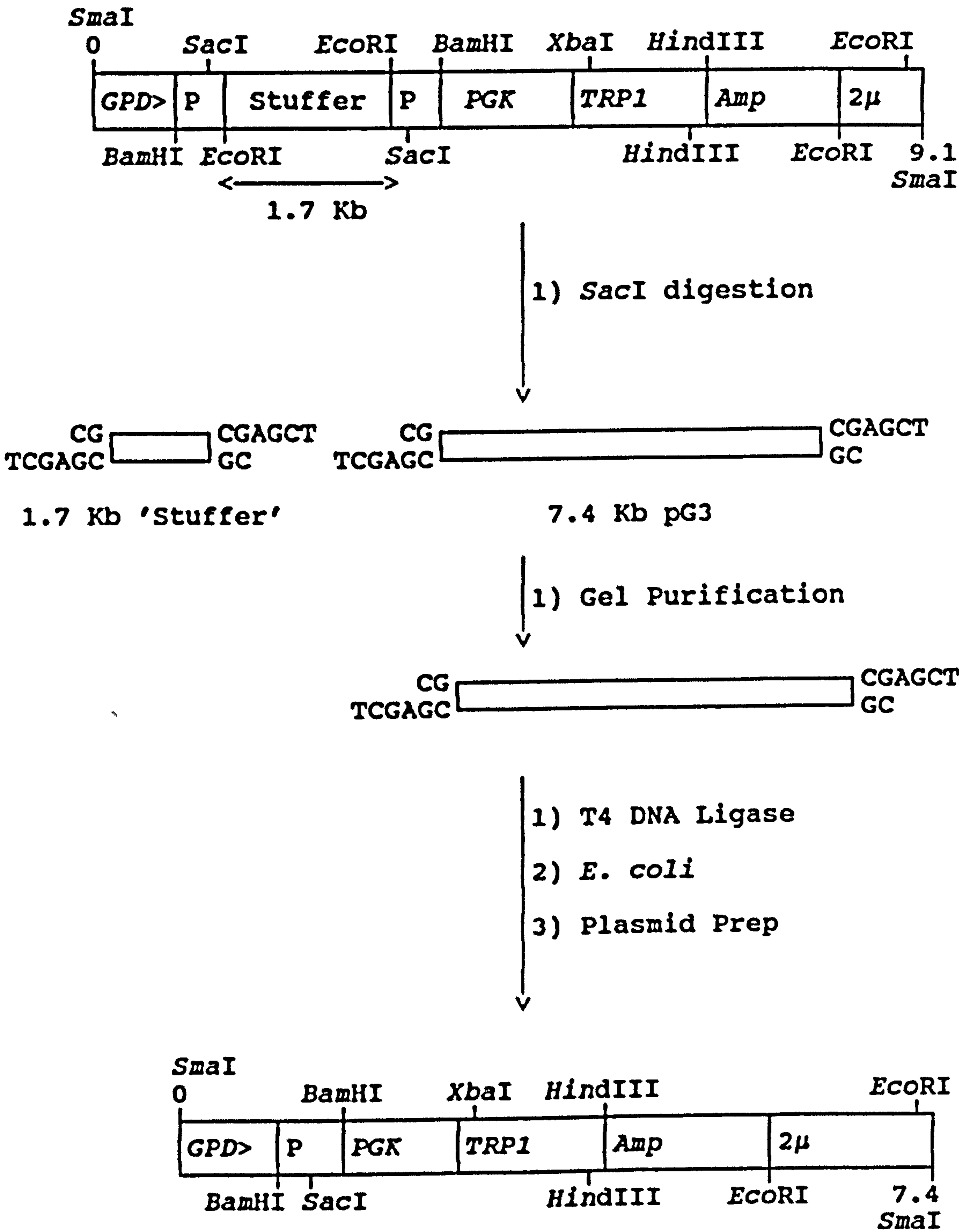
- 1) SacI Digestion
- 2) KpnI Digestion





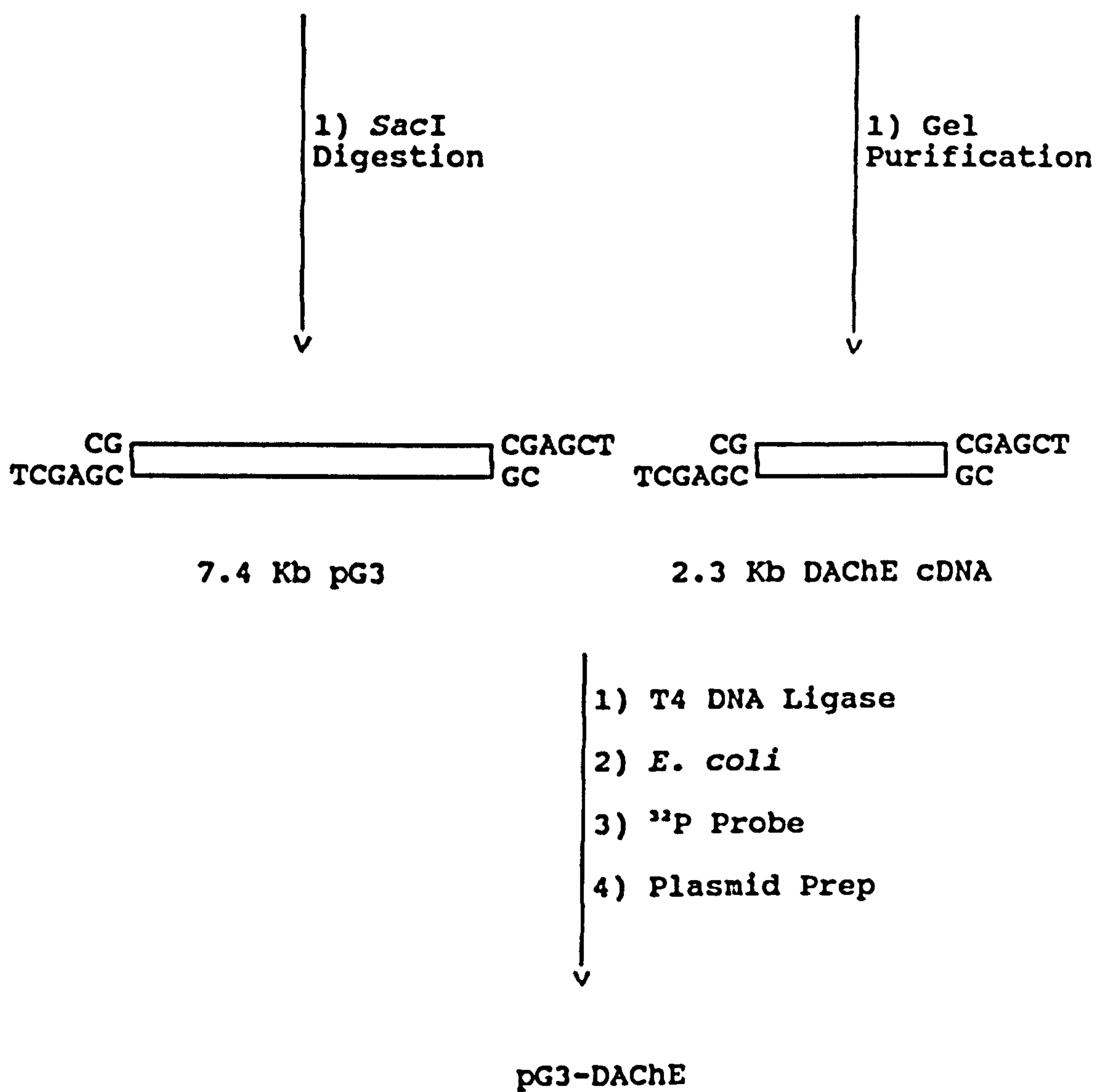
# Strategy for Subcloning into Plasmid pG3

## Preparation of Vector



## Strategy for Subcloning into Plasmid pG3

### Construction of pG3-DACHE





to excise the 2.3 Kb gene fragment on a simple SacI digest without any linker ligation steps. The fragment was ligated into pG3 (Table 4.5.). Colony lifts were probed using the <sup>32</sup>P labelled EcoRI-SacI fragment. Minipreps were carried out on colonies in the vicinity of positive signals. Finally, ≈500 colonies were picked, spotted in a grid system using a large library agar plate, regrown overnight at 37°C, and lifted and screened with the <sup>32</sup>P labelled probe. Each positive clone identified was isolated and a quick plasmid prep performed on it.

Table 4.3. Ratios of vector:insert used in subcloning into phosphatased pG3.

Plate	Vector (ng)	Insert (ng)	Units of T4 DNA Ligase (New England Biolabs)
1	≈200	≈80	40
2	≈200	≈80	4
3	≈100	≈100	40
4	≈100	≈100	4
5	≈200	≈120	40
6	≈200	≈120	40
7	≈200	0	40
8	≈200 <sup>UP</sup>	0	40
9	0	0	0

UP - Unphosphatased vector control ligation.

Table 4.4. Ratios of vector:insert used in subcloning into unphosphatased pG3.

Plate	Vector (ng)	Insert (ng)	Units of T4 DNA Ligase (New England Biolabs)
1	≈100	≈100	40
2	≈100	≈100	4
3	≈200	≈200	40
4	≈200	≈200	4
5	≈200	0	40
6	0	0	0

Table 4.5. Ratios of vector:insert used in subcloning into unphosphatased pG3 following cloning of insert via pUC19.

Plate	Vector (ng)	Insert (ng)	Units of T4 DNA Ligase (New England Biolabs)
1	≈100	≈200	40
2	≈100	≈200	40
3	≈100	≈200	40
4	≈100	0	40
5	0	0	0

4.2.3. Subcloning into pPIC9

In order to increase the chances of successful secretion it was decided to delete the first 38 amino acids from the cDNA using PvuI. These first 38 amino acids make up the



hydrophobic signal peptide of 'pre-acetylcholinesterase' as described by Hall and Spierer (1986). This hydrophobic region, required for transport across the ER membrane *in vivo*, may have resulted in secretion from yeast. However, the successful secretion of a heterologous protein from yeast often requires the attachment of a suitable yeast secretion signal. pPIC9 contains the  $\alpha$ -factor secretion signal sequence. It was necessary to fuse the DACH<sub>E</sub> cDNA in frame with this sequence. The subcloning strategy and precise in frame fusion proposed are illustrated in Figures 4.5 and 4.6. A double stranded oligonucleotide was designed containing restriction sites for PvuI and XhoI and an endogenous signal cleavage site (necessary if cloning into the XhoI site of pPIC9 because the XhoI site resides upstream from the cleavage signal). pKS-DACH<sub>E</sub> was initially cut with PvuI and the gene fragment gel purified. The oligonucleotide was cut with PvuI, recovered using ultracentrifugation (2.15.2), and ligated to the *D. melanogaster* cDNA at the PvuI sites.

Approximately 100ng of purified oligonucleotide was ligated with approximately 200ng of purified cDNA in a final volume of 40 $\mu$ l with 40 Units (New England Biolabs) of T4 DNA ligase for 48 hr at 4°C. 4 ligations were set up. The ligase was inactivated by heating to 65°C for 15 min and the cDNA + ligated oligonucleotide cut with XhoI and repurified from solution through Spinbind. The success of the pG3 subcloning method through pUC19 led to the decision to initially subclone into the XhoI site of Bluescript KS+ (Stratagene), screen for inserts with X-gal, and then confirm the gene's orientation before cutting out on a XhoI-NotI fragment and ligating into a pPIC9 vector cut with XhoI and NotI. This meant that any positive clones isolated would contain the gene in the correct orientation.

Figure 4.5. A double stranded oligonucleotide containing PvuI and XhoI sites was designed for subcloning of the DAcHE gene into the secretion plasmid pPIC9. PvuI digestion of pKS-DACHE results in a gene fragment minus the first 38 amino acids of 'pre-acetylcholinesterase' (Hall and Spierer, 1986).



Strategy for Subcloning into pPIC9

Double Stranded Oligonucleotide

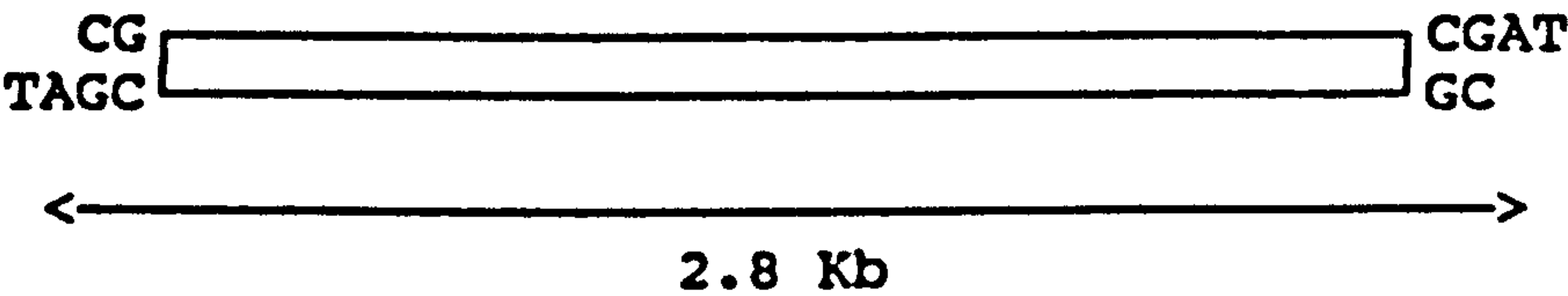
Clamp | XhoI Signal |  
GCGCGCGCTCGAGAGAGAGGTCATCGATCGCGCGCGC  
CGCGCGCGAGCTCTCTCTCCAGTAGCTAGCGCGCGCG  
| PvuI | Clamp

- ↓
- 1) PvuI Digestion  
2) Phenol:Chloroform ETOH ppt  
3) Ultracentrifugation

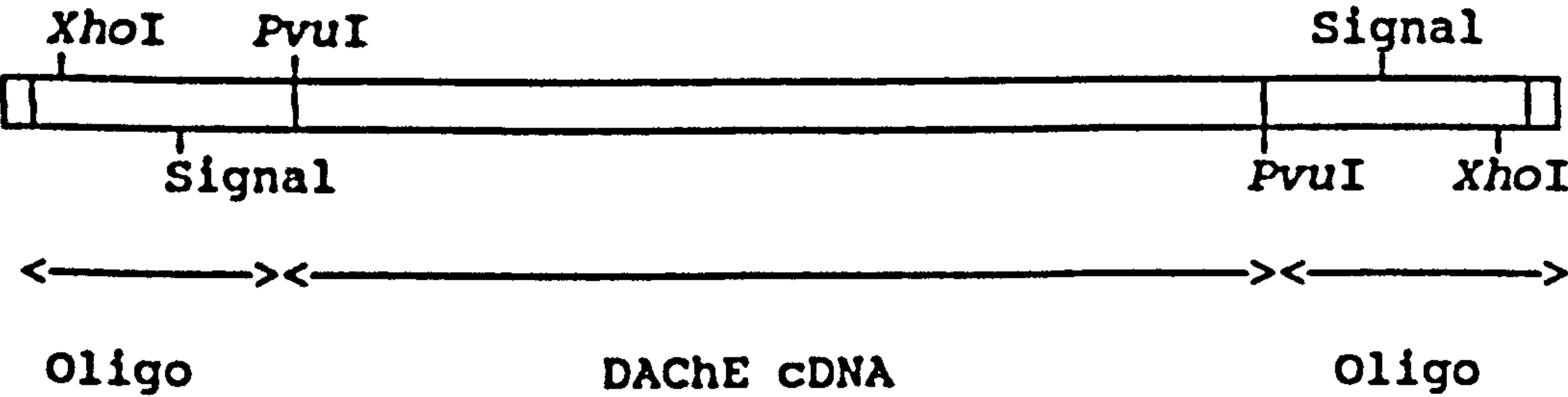
GCGCGCGCTCGAGAGAGAGGTCATCGAT  
CGCGCGCGAGCTCTCTCTCCAGTAGC

+

D. melanogaster AChE cDNA PvuI Fragment



- ↓
- 1) T4 DNA Ligase



↓

Signal  
TCGAGAGAGAG  CTCTCTC  
CTCTCTC GAGAGAGAGCT  
XhoI Sticky End

**Bluescript KS+ Digested with XhoI**

↓

Signal  
 TCGAGAGAGAG  
 CTCTCTC  
 XhoI

DAcHE cDNA

GC  
 CGCCGG  
 NotI

**pPIC9 Cut with XhoI and NotI**

**<----->**

- 1) T4 DNA Ligase
- 2) *E. coli*
- 3) <sup>32</sup>P Probe
- 4) Miniprep

**pPIC9-DAChE**



Figure 4.6. The proposed in-frame fusion between the  $\alpha$ -signal peptide and the DAcHE cDNA sequence following deletion of the native signal peptide by digestion with *PvuI*.

\* Val<sup>39</sup> the first amino acid of the mature DAcHE protein and the following two amino acids Ile<sup>40</sup> and Asp<sup>41</sup> are encoded in the designed oligonucleotide. The codon for Asp<sup>41</sup> is reformed on splicing into the *PvuI* site of the DAcHE cDNA at position 1206 (Figure 4.1). Cloning the spliced oligonucleotide and DAcHE cDNA into the *XhoI* site of pPIC9 then places the fragment in frame with the  $\alpha$ -factor peptide encoded by the upstream sequence in pPIC9.

mRNA AOX1 5' end

ACAGCAATATATAAACAGAAGGAAGCTGCCCTGTCTTAAACCTTTTTTTTTTATCATCATTAT

TAGCTTACTTTCATAATTGCGACTGGTTCCAATTGACAAGCTTTTGATTTTAACGACTTTTA

αF start

Met Arg Phe

ACGACAACCTTGAGAAGATCAAAAAACAACCTAATTATTCGAAGGATCCAAACG ATG AGA TTT

Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT

Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala

GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT

Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe Asp Val Ala

GAA GCT GTC ATC GGT TAC TCA GAT TTA GAA GGG GAT TTC GAT GTT GCT

Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn

GTT TTG CCA TTT TCC AAC AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT

XhoI

Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val Ser Leu Glu

ACT ACT ATT GCC AGC ATT GCT GCT AAA GAA GAA GGG GTA TCT CTC GAG

\* PvuI

Arg Glu Val Ile Asp Arg Leu

AGA GAG GTC ATC GAT CGC CTG.....

DACHE cDNA start

Signal Cleavage



The following ligations of *Xho*I cut Bluescript KS+ with *Xho*I cut DACH<sub>E</sub> cDNA + oligonucleotide were set up:

Table 4.6. Ratios of vector to insert used in subcloning into Bluescript KS+ prior to pPIC9.

Plate	Vector (ng)	Insert (ng)	Units of T4 DNA ligase (New England Biolabs)
1	200	100	40
2	200	100	40
3	100	50	40
4	200	0	40

Ligations were transformed directly into *E. coli* and plated onto X-gal. Incubation was overnight at 37°C.

### 4.3. Results

#### 4.3.1. Subcloning into p2UG

Methylated pKS-DACH<sub>E</sub> was digested with *Bam*HI as a control for methylation and this is shown in Figure 4.7.b. It can be seen that the methylated sample treated with *Bam*HI remains uncut. The methylated *Sma*I-*Hinc*II fragment of 3.3 Kb is shown in Figure 4.7.c. The *Bam*HI digest of p2UG giving a linear vector of 6.0 Kb is shown in Figure 4.7.d. Following ligation of insert and vector the ligations were transformed into *E. coli*, plated on L. B. Medium with ampicillin, and incubated at 37°C. Table 4.7. gives the results for one set of ligations. It can be seen that the number of colonies recovered indicates that the phosphatasing step had worked efficiently but the unphosphatased vector control (Plate 5) of 45 colonies suggests that either the original ligation or the transformation had been very inefficient.

Figure 4.7.a. Restriction digests of pKS-DAC<sub>H</sub>E (6.2 Kb).

- Lane 1 - 1 Kb DNA ladder (Gibco-BRL)
- 2 - Uncut pKS-DAC<sub>H</sub>E
- 3 - *Eco*RI-*Sac*I digest showing the 1.5 Kb fragment used as a probe. This fragment spans a large section of the coding region of the gene
- 4 - *Sac*I digest
- 5 - *Eco*RI digest

Figure 4.7.b. A test digest of pKS-DAC<sub>H</sub>E with *Bam*HI following methylation of endogenous sites with *Bam*HI methylase. The methylated sample remains uncut.

- Lane 1 - Lambda *Hind*III ladder
- 2 - *Bam*HI digest of unmethylated pKS-DAC<sub>H</sub>E
- 3 - *Bam*HI digest of methylated pKS-DAC<sub>H</sub>E

Figure 4.7.c. 3.3 Kb *Sma*I-*Hinc*II fragment of the DAC<sub>H</sub>E gene following methylation, excision, and subsequent gel purification.

- Lane 1 - Lambda *Hind*III ladder
- 2 - DAC<sub>H</sub>E fragment

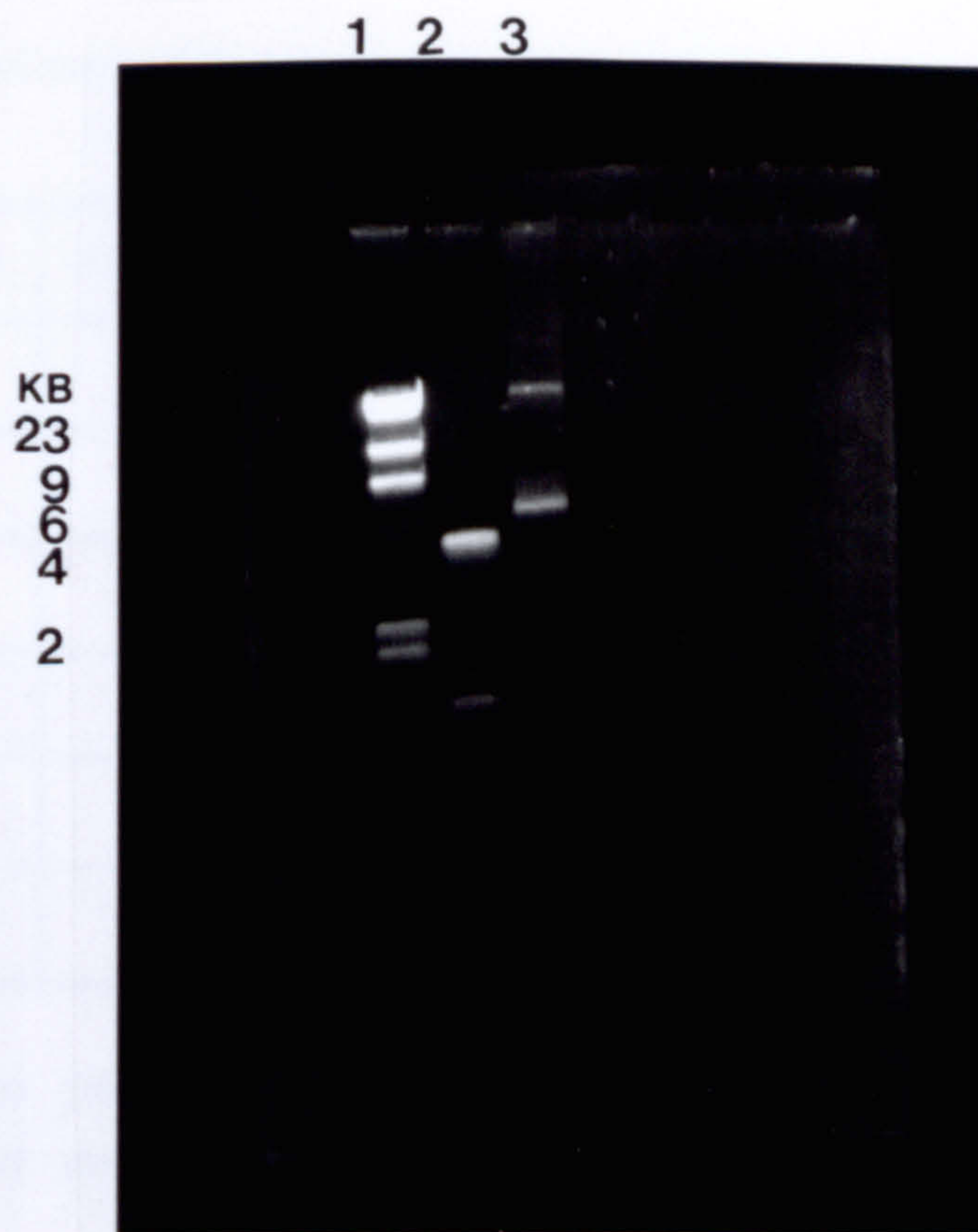
Figure 4.7.d. *Bam*HI digest of plasmid p2UG giving a linear vector of 6.0 Kb.

- Lane 1 - Lambda *Hind*III ladder
- 2 - *Bam*HI digest of p2UG
- 3 - *Bam*HI digest of p2UG

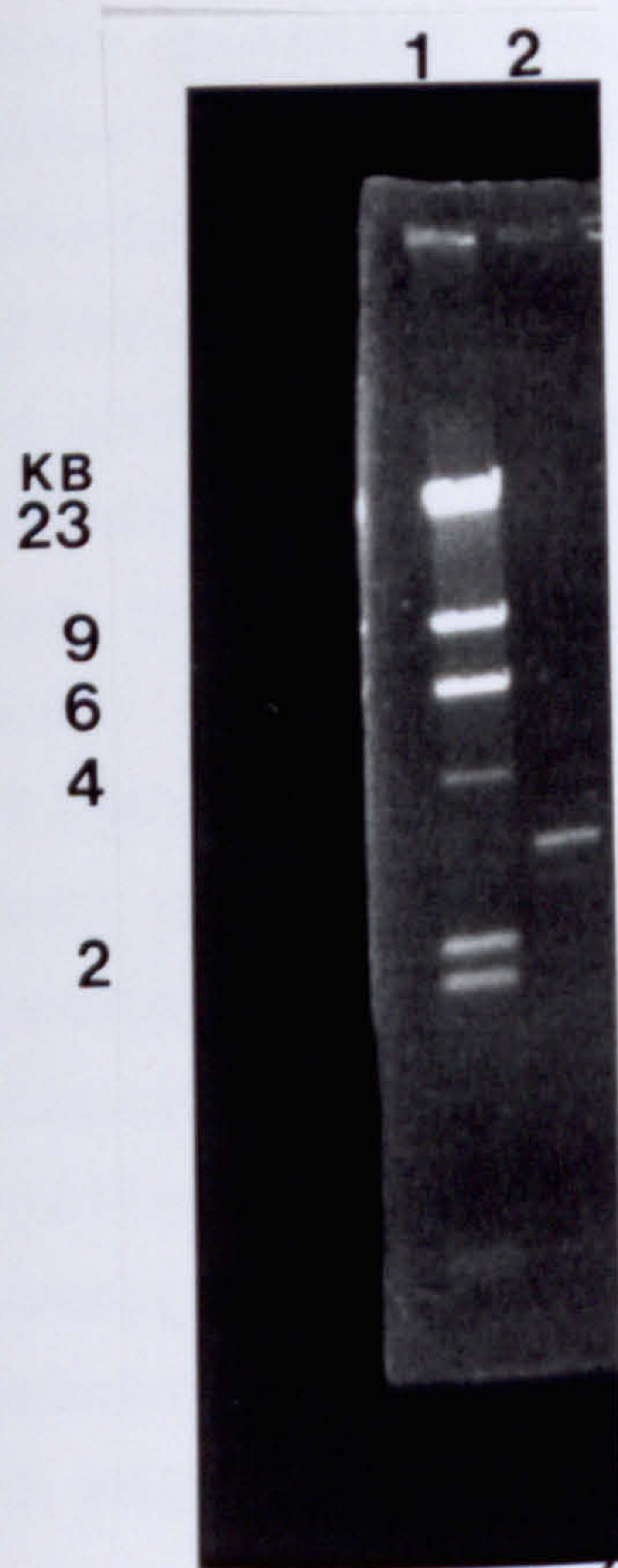




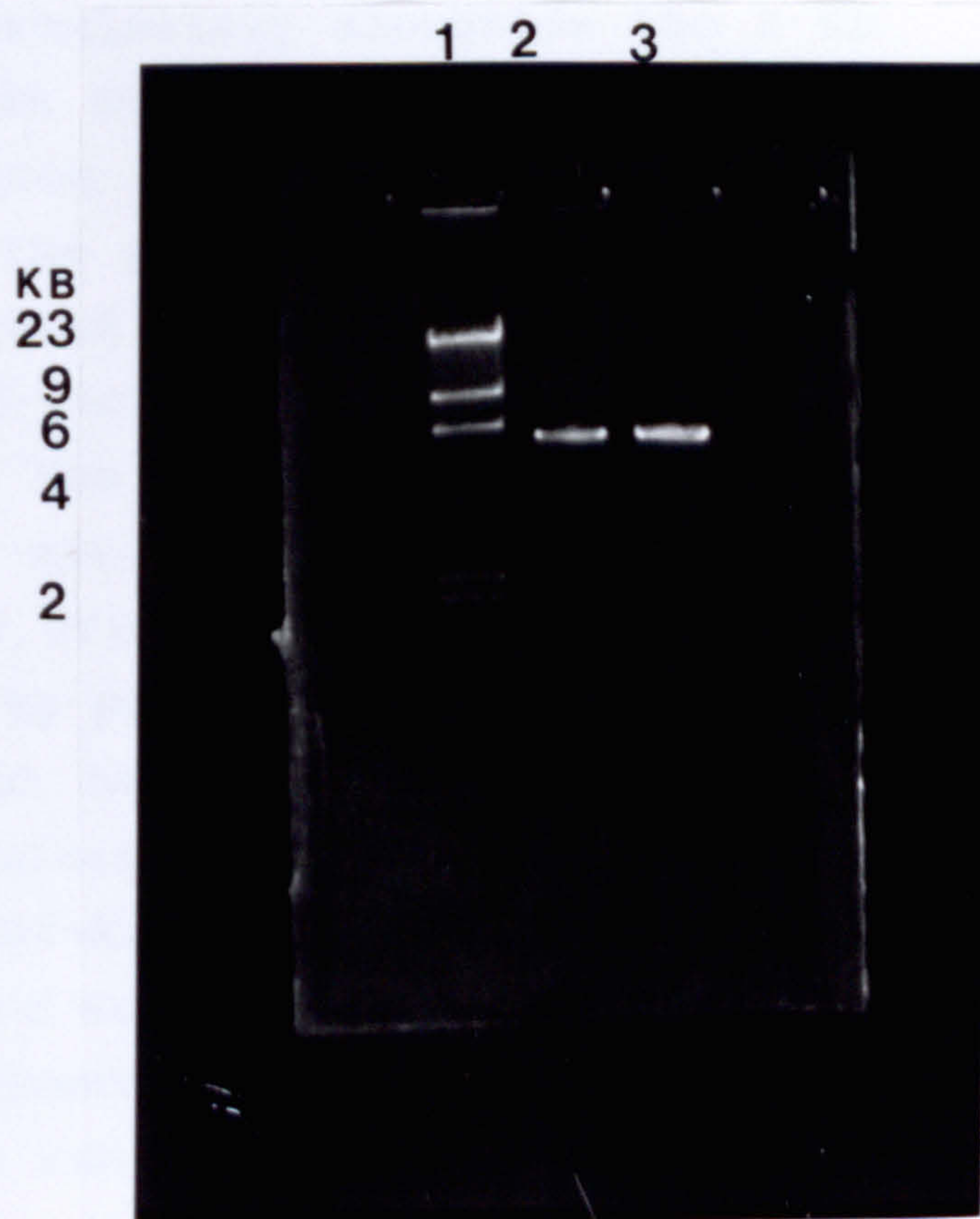
A



B



C



D



Table 4.7. Transformants obtained for a set of ligations into phosphatased p2UG.

Plate	N° of Colonies	Plate	N° of Colonies
1a*	17	1b*	0
2a	11	2b	0
3a	13	3b	0
4a	0	4b	0
5a	45	5b	0
6a	0	6b	0

\* 'a' refers to a spread of the pellet of transformants.

\* 'b' refers to a second spread using the same spreader.

10 colonies were picked from plates 1a, 2a, and 3a and minipreps performed on them. Figure 4.8.a. shows the results of the 10 minipreps from plate 3a (the preps from the other plates ran similarly). It can be seen that the uncut plasmids have run approximately alongside the 2 Kb marker. These DNA fragments were too small to contain inserts. Subsequent digestion with *EcoRI* and *XbaI* with suitable controls revealed the plasmids to be Bluescript KS+ with *BamHI* linkers religated on itself (Figure 4.8.b). *SmaI-HincII* digestion of pKS-DAC<sub>H</sub>E removes the *EcoRI* site from the polylinker whereas the *XbaI* site remains. Thus *EcoRI* digestion showed the miniprep Bluescript to be a religated fragment from the original *SmaI-HincII* digest: *EcoRI* did not cut the miniprep plasmids but linearized the Bluescript control; *XbaI* cut both miniprep plasmids and Bluescript control. Contamination of the original gel purified sample of the *SmaI-HincII* gene fragment with Bluescript when the DAC<sub>H</sub>E band was cut from the gel was the probable cause. The two fragments of 3.3 Kb DAC<sub>H</sub>E gene and 2.9 pKS+ given by the digest run quite close together.



Figure 4.8.a. Plasmid minipreps resulting from a set of ligations into phosphatased p2UG. No inserts were obtained. Lane 12 is a control lane containing uncut p2UG.

Lane 1	-	Lambda <i>Hind</i> III ladder
2 to 11	-	Uncut minipreps
12	-	Uncut p2UG
13	-	Lambda <i>Hind</i> III ladder

Figure 4.8.b. Restriction digests identifying plasmids as Bluescript KS+ with *Bam*HI linkers religated on itself. Contamination with similar size vector had occurred during the initial excision and purification of the gene, circumvented in subsequent subcloning attempts by additional digestion with *Kpn*I.

Lane 1	-	Lambda <i>Hind</i> III ladder
2	-	Uncut control pKS+
3	-	<i>Eco</i> RI digest of control pKS+
4 to 8	-	<i>Eco</i> RI digest of minipreps
9	-	<i>Xba</i> I digest of control pKS+
10 to 14	-	<i>Xba</i> I digest of minipreps

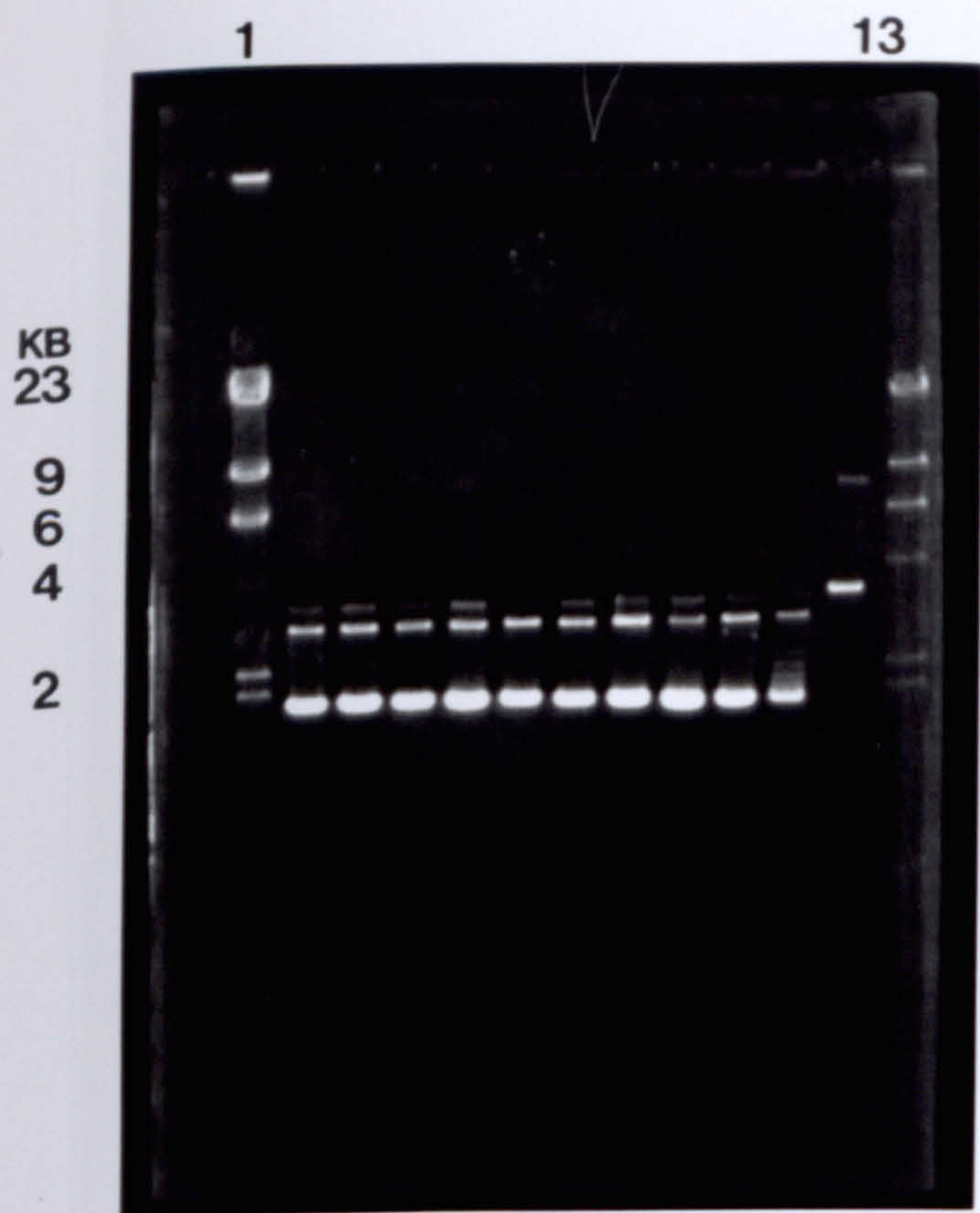
Figure 4.8.c. Plasmid minipreps resulting from a second set of ligations into unphosphatased p2UG probed using ECL. Lane 8 is a control lane containing uncut p2UG. No inserts were obtained.

Lane 1	-	Lambda <i>Hind</i> III ladder
2 to 7	-	Uncut minipreps
8	-	Uncut p2UG

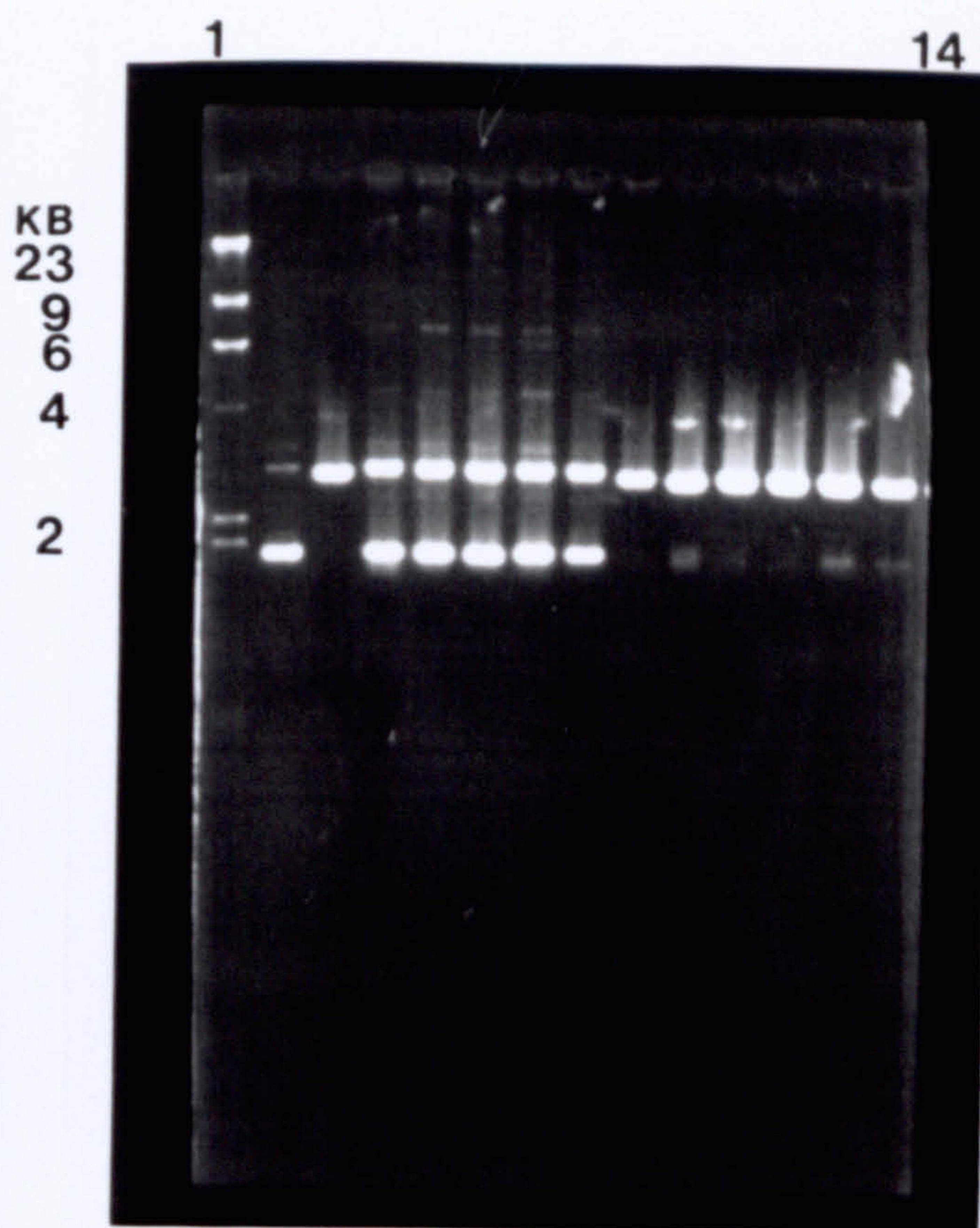
Figure 4.8.d. Restriction digests confirming minipreps as p2UG religated on itself.

Lane 1	-	Lambda <i>Hind</i> III ladder
2 to 5	-	<i>Eco</i> RI digest of minipreps
6	-	<i>Eco</i> RI digest of control pKS+
7	-	<i>Eco</i> RI digest of pKS-DAC <sub>H</sub> E
8	-	<i>Eco</i> RI digest of control p2UG

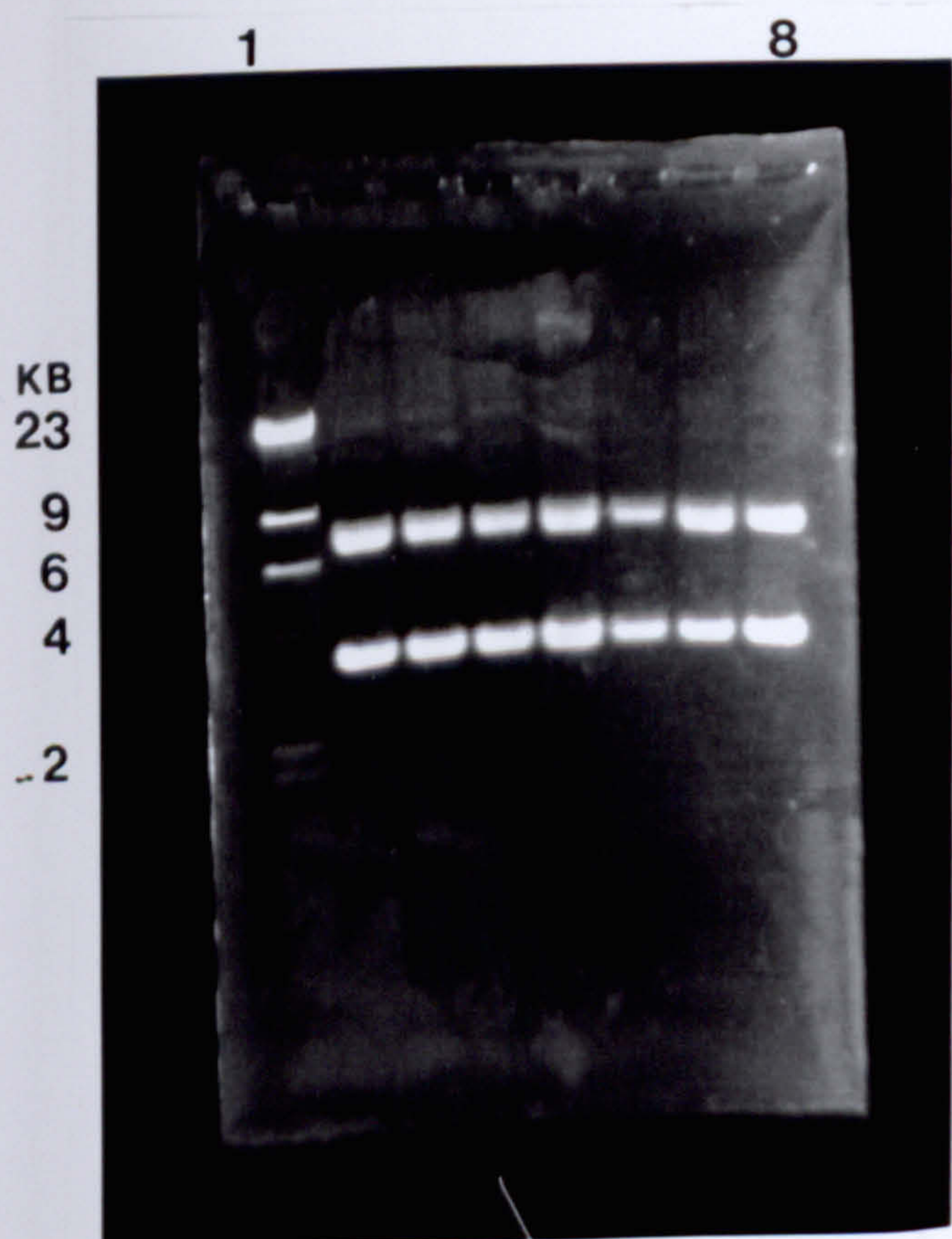




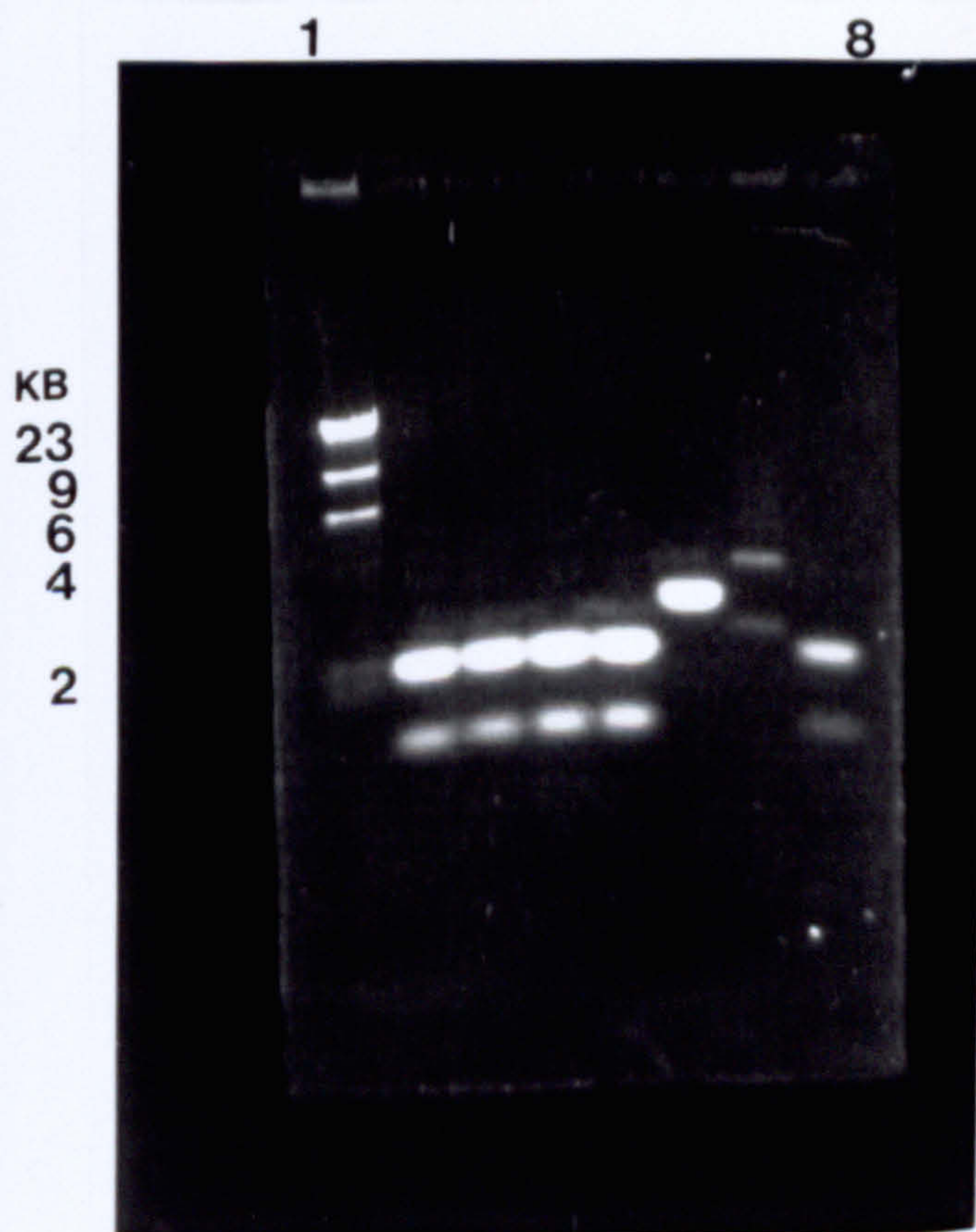
A



B



C



D



The subcloning was then repeated using *KpnI* in addition to *BamHI* at the linker digestion step to prevent the recircularization of any contaminating pKS+. The *KpnI* site in the Bluescript polylinker lies upstream of the *HincII* site and subsequent digestion creates incompatible ends on any contaminating pKS+. Phosphatasing of the vector arms caused problems in the original ligations (the reason for this remains unclear) and therefore, in a second set of ligations, the insert was ligated into an unphosphatased p2UG and subsequently probed using the 1.5 Kb *EcoRI-SacI* fragment labelled by ECL (Amersham). A very low number of transformants was recovered (Table 4.8.).

Table 4.8. Transformants obtained for a set of ligations into unphosphatased p2UG.

Plate	N° of Colonies	Plate	N° of Colonies
1a	61	1b	14
2a	≈100	2b	9
3a	≈100	3b	26
4a	≈100	4b	25
5a	0	5b	0

An additional control transformation using pUC19 performed at the same time resulted in >1000 transformants per pellet plated. This indicates that transformation procedures using DH5α *E. coli* were efficient. The approximate number of unphosphatased p2UG transformants obtained (mean ≈ 87) correlates with the low number recovered from the unphosphatased vector control (plate 5a) in the original subcloning (Table 4.7). The reason for p2UG transforming so poorly was unclear. The first probing using the ECL kit on colony lifts from plates 1a - 3a was unsuccessful. A possibility was that the probe concentration of 2ng/ml was

too low. In a second probing the concentration was increased to 10ng/ml but a 1 min exposure gave no results. Exposure overnight resulted in a possible positive signal on the filter from plate 1a. Seven colonies in the area of the signal were picked with sterile cocktail sticks and minipreps carried out. The results are shown in Figure 4.8.c and d. Subsequent digestion with *EcoRI* showed the preps to be p2UG ligated on itself.

#### 4.3.2. Subcloning into pG3

A single *SacI* restriction site was produced in vector pG3 by excision of the 'stuffer' fragment and subsequent religation (Figure 4.9.a). It can be seen from lanes 5 and 7 that a *SacI* digest of the vector religated following excision of the stuffer fragment gives a single band of 7.4 Kb. Figure 4.9.b illustrates restriction digests performed on pKS-DACH<sub>E</sub>. *HincII* digestion (lane 3) gives a linear fragment of 6.2 Kb. *SacI* digestion (lane 5) gives two fragments of 5.2 Kb and 1.0 Kb. Lane 4 illustrates the *HincII*-*SacI* digestion used in the subcloning. Three fragments are visible: 2.3 Kb gene, 1.0 Kb gene, and 2.9 Kb vector. In lanes 3-5 there is also some undigested pKS-DACH<sub>E</sub> at approximately 4 Kb. Figure 4.9.c. shows the excision of the 2.3 Kb DACH<sub>E</sub> gene fragment following linearization with *HincII*, ligation of *SacI* linkers, and subsequent digestion with *SacI*. Figure 4.9.d. shows final preparation of the gene fragment following digestion of linkers and subsequent purification (lanes 4 and 5). The same gel also shows final preparations of pG3 after *SacI* digestion and purification. Lane 2 is unphosphatased vector and lane 3 pG3 after treatment with CIAP.

The transformation results from the set of ligations using pG3 phosphatased with SAP are shown in Table 4.9.



Figure 4.9.a. *SacI* digests of pG3 showing excision of the 1.7 Kb 'stuffer' fragment to create a modified vector with a single *SacI* site.

Lane 1 - Lambda *HindIII* ladder

2 - Uncut pG3

3 - *SacI* digest of original pG3

4 - Uncut modified pG3

5 - *SacI* digest of modified pG3

6 - Uncut modified pG3

7 - *SacI* digest of modified pG3

Figure 4.9.b. Restriction digests illustrating cuts used in the pG3 subcloning strategy. See text for details.

Lane 1 - Lambda *HindIII* ladder

2 - Uncut pKS-DAC<sub>H</sub>E

3 - *HincII* digest of pKS-DAC<sub>H</sub>E

4 - *HincII-SacI* digest of pKS-DAC<sub>H</sub>E

5 - *SacI* digest of pKS-DAC<sub>H</sub>E

Figure 4.9.c. Excision of the 2.3 Kb gene fragment following the attachment of linkers and *HincII-SacI* digestion. Possible contamination in the final ligation with the 2.9 Kb vector seen here was circumvented by additional digestion with *KpnI* during the subcloning. Middle lane - 1 Kb ladder.

Figure 4.9.d. Final preparations of 2.3 Kb DAC<sub>H</sub>E gene fragment and *SacI* linearized 7.4 Kb pG3.

Lane 1 - 1 Kb ladder

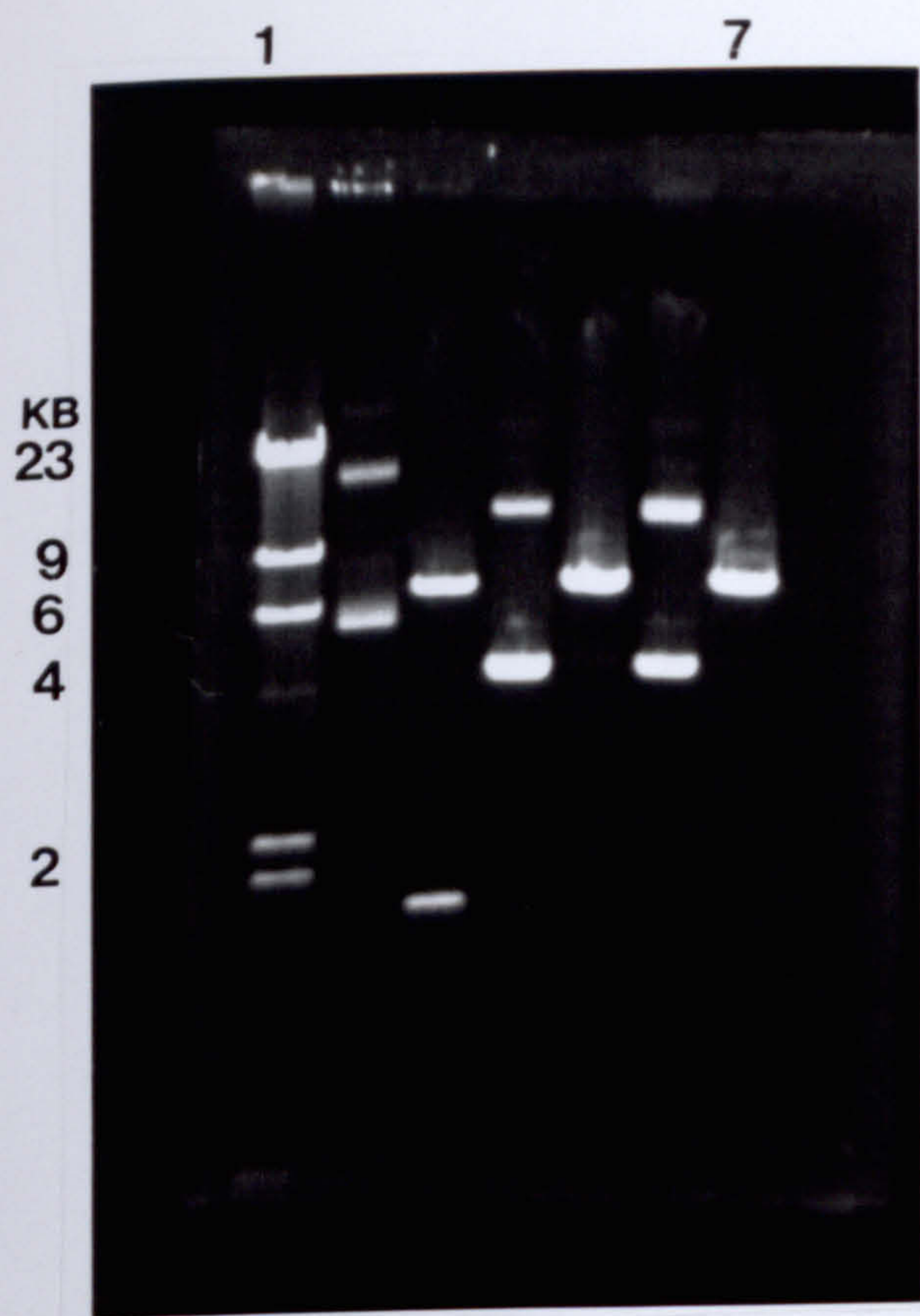
2 - pG3

3 - pG3

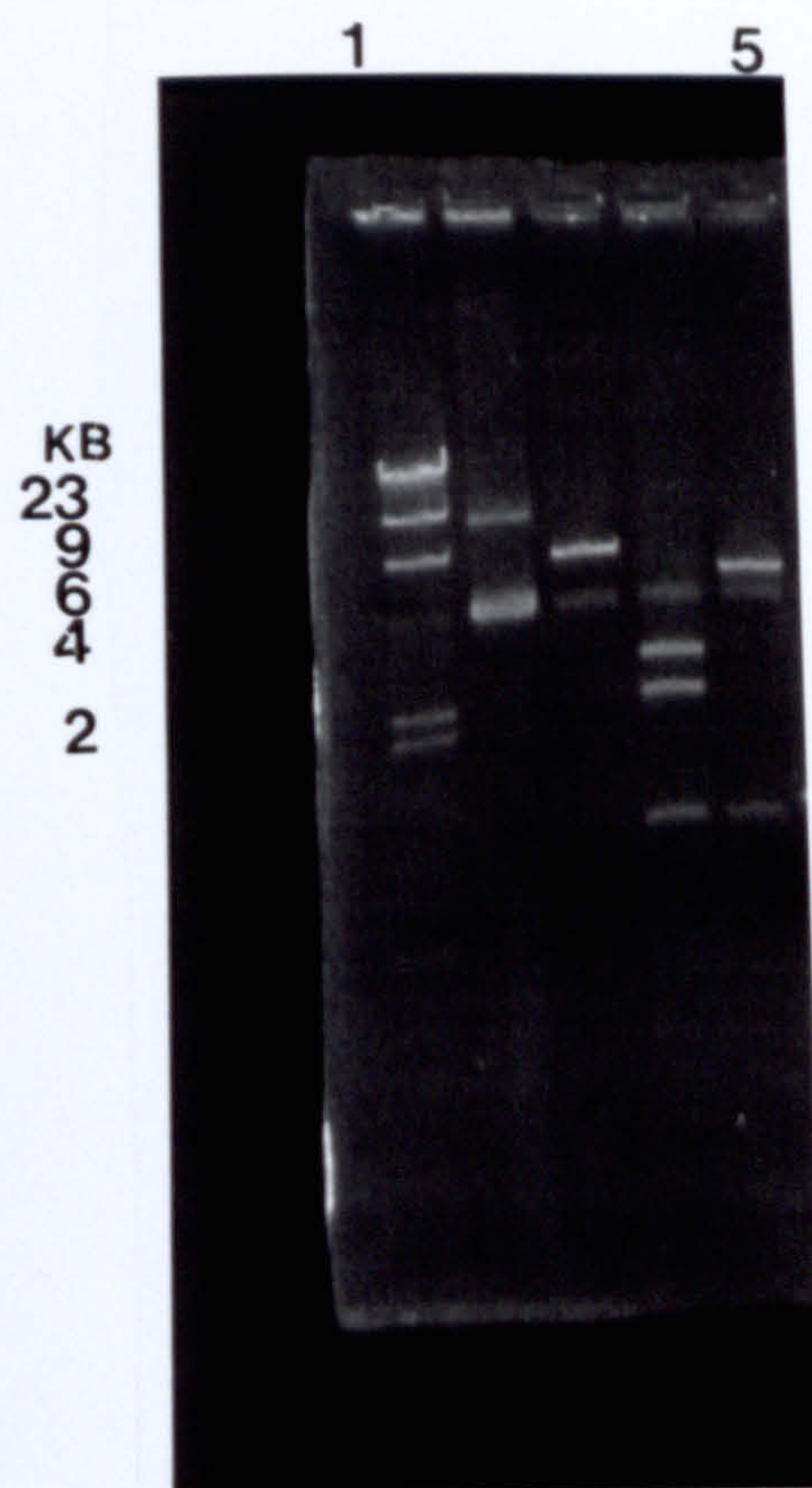
4 - DAC<sub>H</sub>E (very faint)

5 - DAC<sub>H</sub>E (very faint)

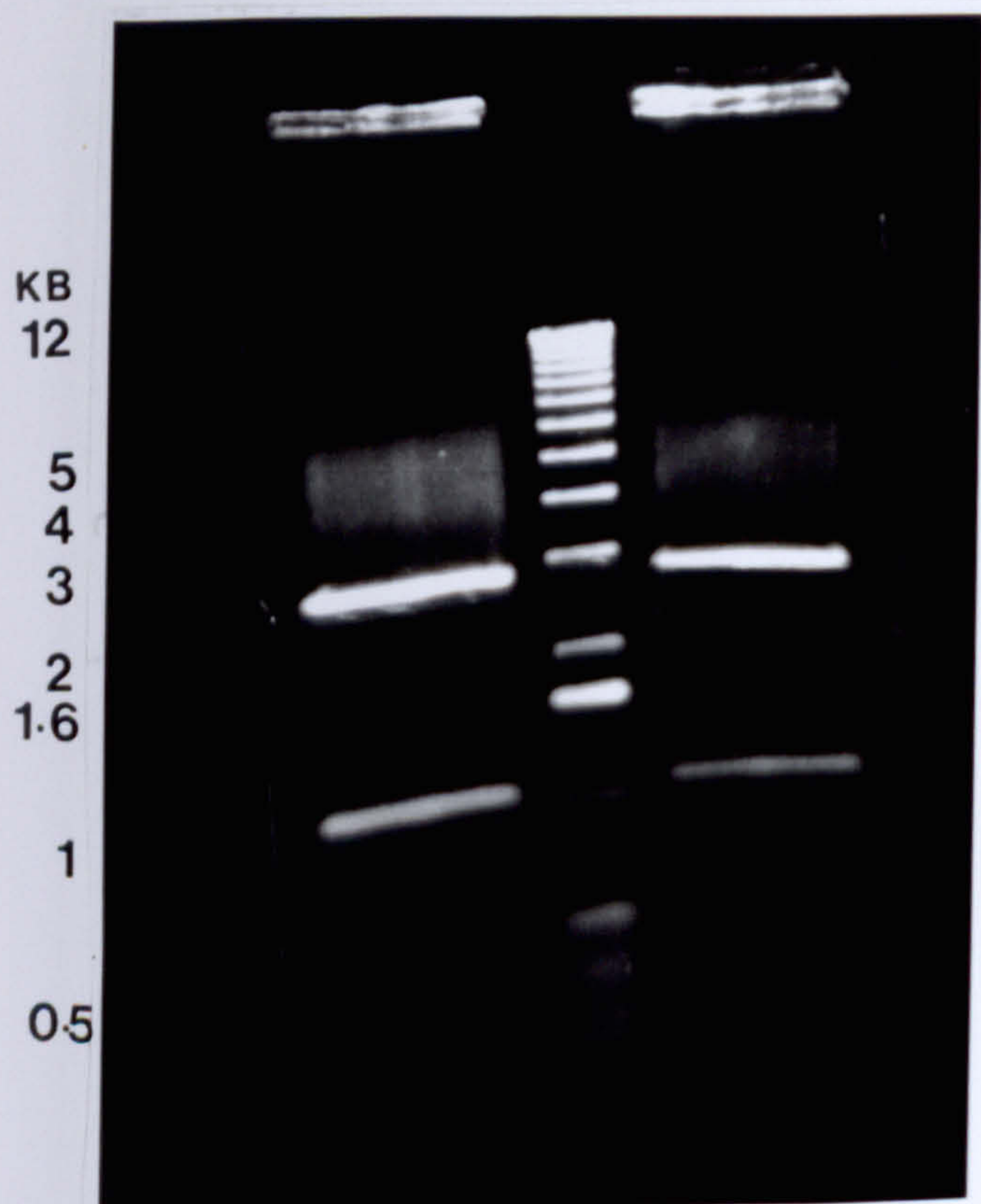




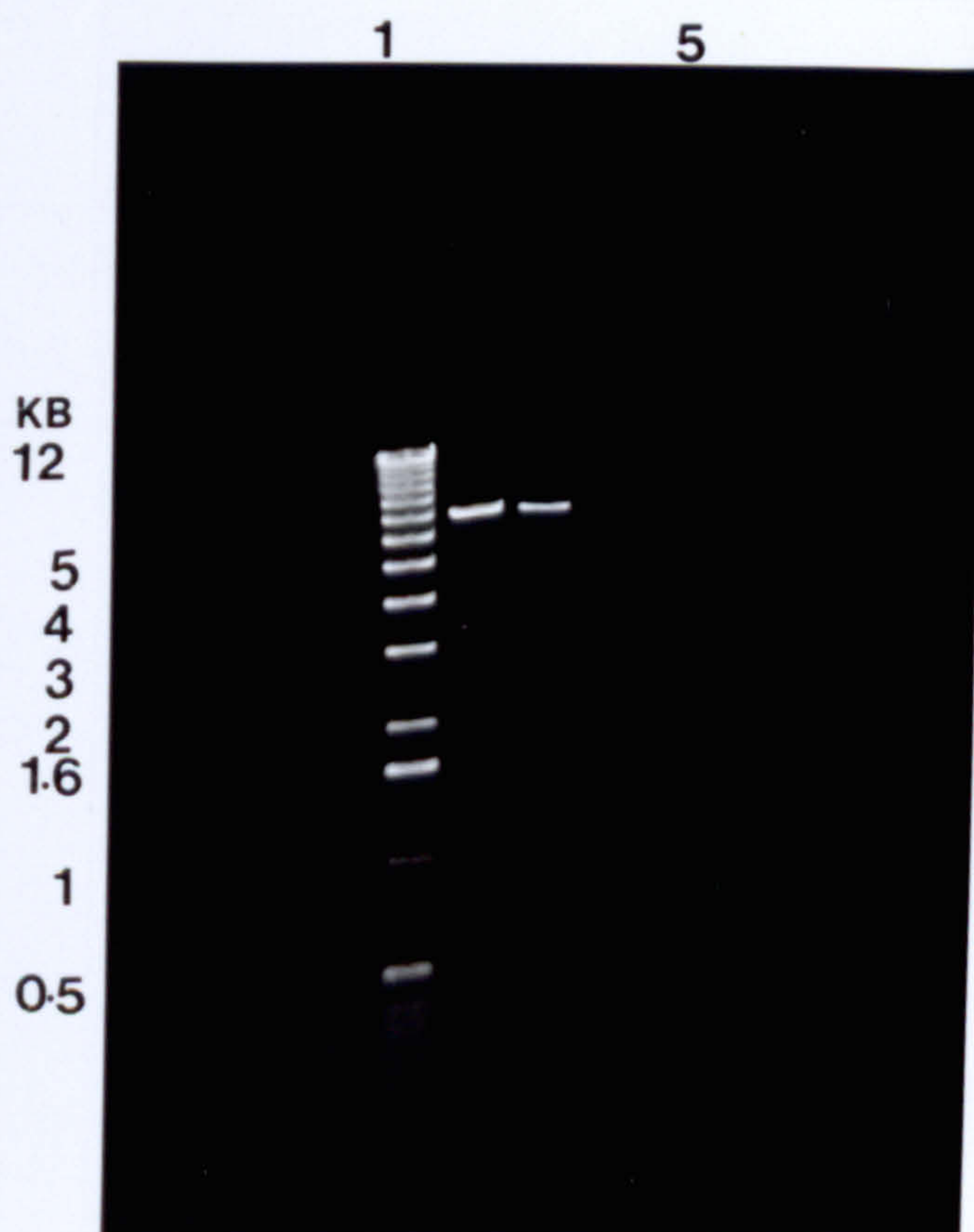
A



B



C



D



All experimental plates contained approximately the same number of colonies as the phosphatased control (Plate 7) with the exception of Plate 6 which was found to have approximately double the number. 20 colonies were picked from this plate and minipreps performed on them. The results are shown in Figure 4.10.a. It can be seen that none appeared to contain an insert. Subsequent digestion of the minipreps with *SacI* showed them to be pG3 religated on itself (Figure 4.10.b.). Plate number 8 contained greater than 1000 colonies as would be expected from the unphosphatased control.

Table 4.9. Transformants obtained for a set of ligations into phosphatased pG3.

Plate	N° Colonies*
1	≈100
2	≈100
3	≈100
4	≈100
5	≈100
6	≈200
7	≈100
8	>1000
9	0

\* N° Colonies obtained are for a 200μl cell pellet.

Good transformations were obtained for a set of ligations using unphosphatased pG3 and the 2.3 Kb DChE gene (>>1000 colonies/plate) but probing of colony lifts with the <sup>32</sup>P labelled *EcoRI-SacI* probe was unsuccessful.

Figure 4.10.a. Plasmid minipreps prepared from plate 6 (see text) resulting from a set of ligations into pG3 phosphatased with CIAP. None of the religated vectors contained an insert.

Lane 1                - 1 kb ladder  
2 to 21           - Uncut miniprep plasmids

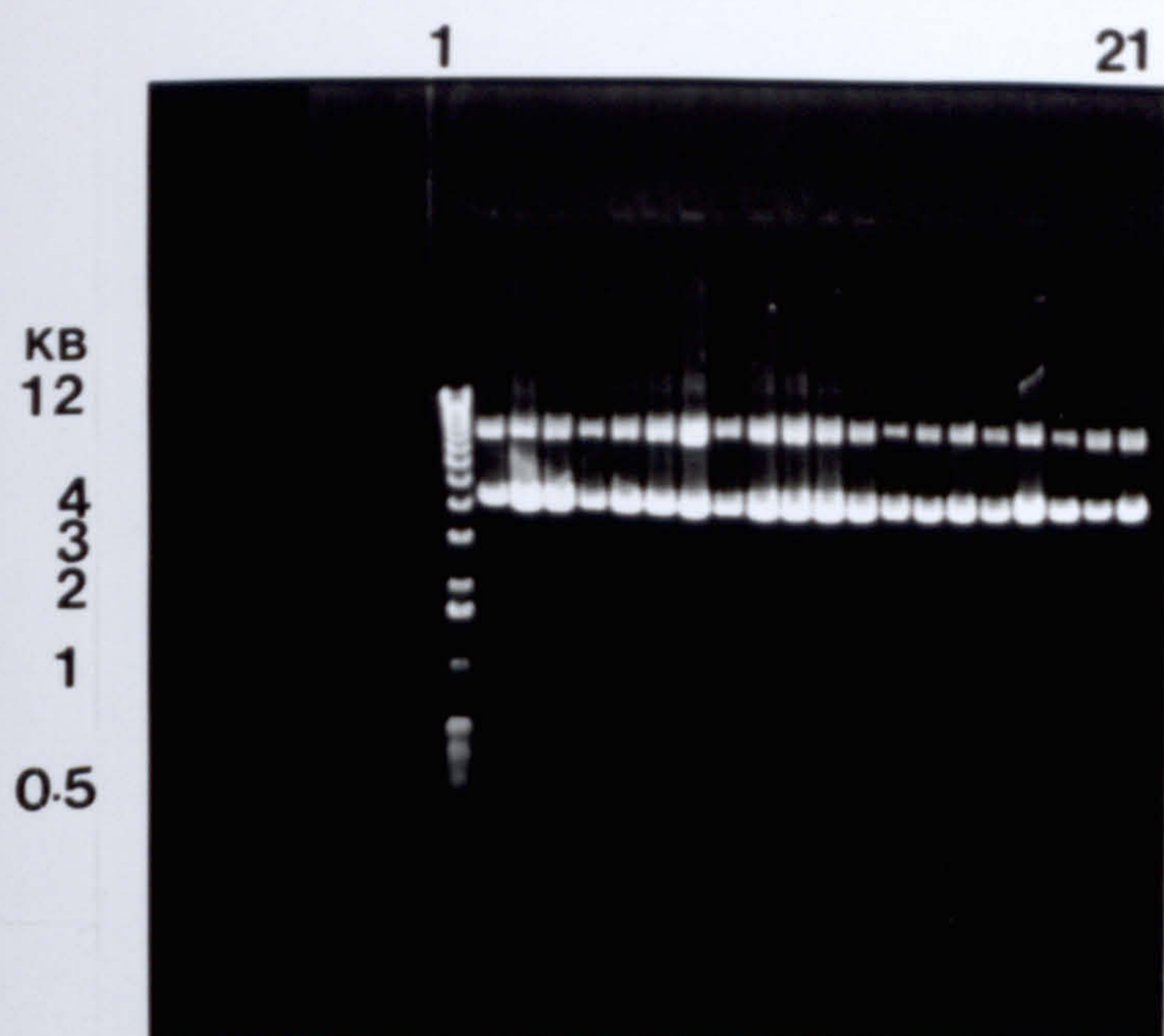
Figure 4.10.b. The minipreps from plate 6 (see text) were pG3 religated on itself.

Lane 1                - 1 Kb ladder  
2 to 6               - *SacI* digested minipreps  
7                      - *SacI* digested pG3 control  
8                      - Uncut pG3 control

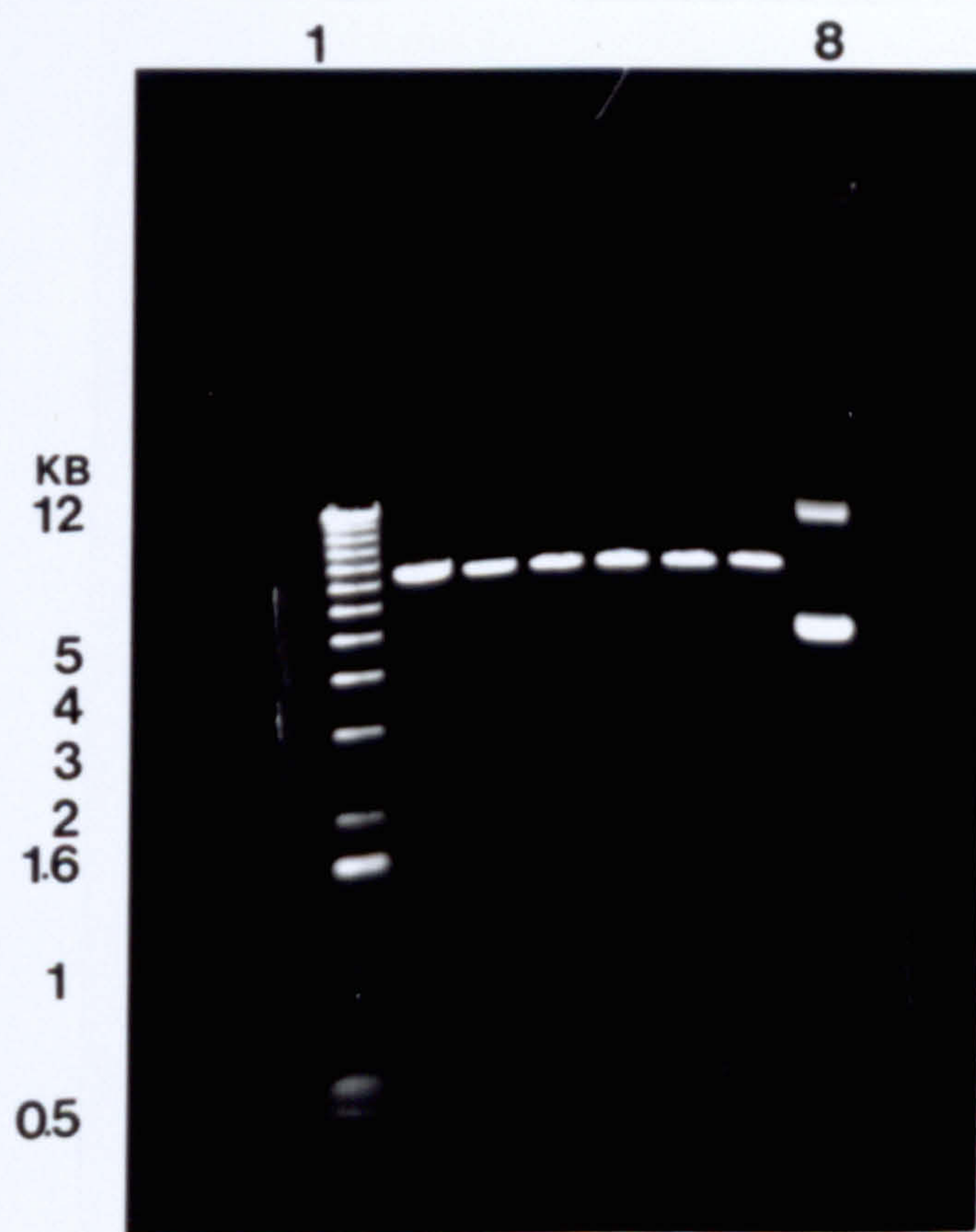
Figure 4.10.c. The DACH gene was eventually ligated into pUC19 during the pG3 subcloning.

Lane 1 - 1 Kb ladder  
2 - Uncut control pUC19  
3 - Uncut miniprep prepared from isolated recombinant  
4 - *SacI* digested control pUC19  
5 - *SacI* digested recombinant (2 fragments : 2.9 Kb pUC19 and 2.3 Kb DACH gene)

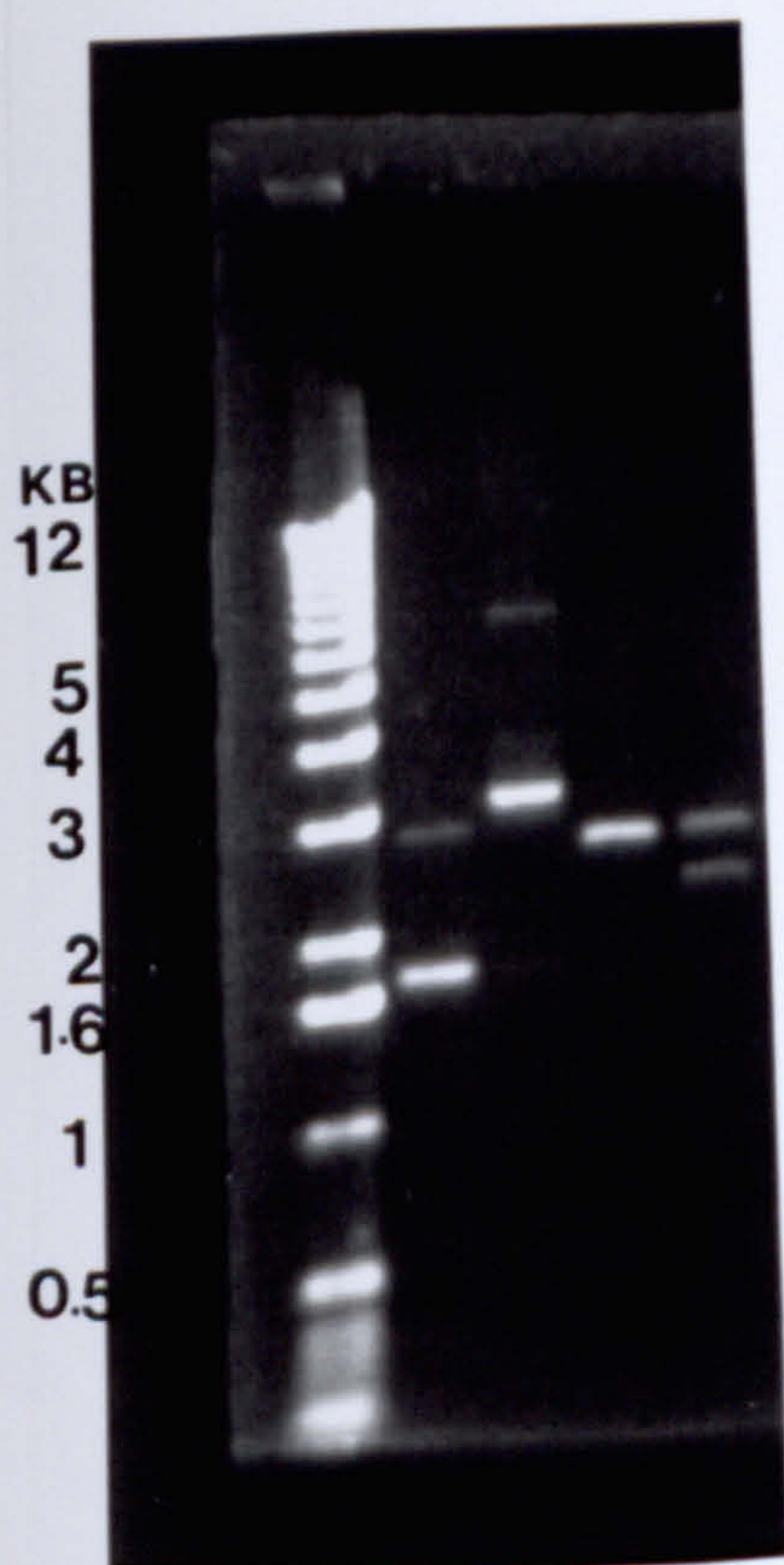




A



B



C



To avoid repetition of linker ligation steps and generate good quantities of insert for repeat ligations 100ng of the 2.3 Kb DChE gene fragment prepared during previous subcloning attempts was used to clone into the SacI site of pUC19 (200ng). Greater than 5000 colonies were obtained per plate with the percentage of white colonies at about 0.5%. 15 white colonies were taken and restreaked on X-gal plates before incubating overnight at 37°C. It was then possible to pick out individual white colonies which were subsequently restreaked again on X-gal and grown overnight at 37°C. A wedge of cells was used from each plate for a miniprep. One of the minipreps appeared to contain the gene and diagnostic cuts confirmed this (Figure 4.10.c). The plasmid was retransformed into *E. coli* and a large scale plasmid prep performed. The 2.3 Kb DChE fragment could then be cut out on a simple SacI fragment and repeatedly ligated with pG3. Table 4.5 illustrates one set of ligations carried out. The number of transformants obtained from each plate is shown below.

Table 4.10. Transformants obtained for a set of ligations into unphosphatased pG3 following cloning of insert via pUC 19.

Plate	N° Colonies*	Plate	N° Colonies**
1a	≈500	1b	≈5000
2a	≈500	2b	≈5000
3a	≈500	3b	≈5000
4a	≈500	4b	≈5000
5a	0	5b	0

\* Spread of 100μl of 1ml *E. coli* transformation.

\*\* Spread of pellet (900μl).



Colony lifts were performed on plates 1a and 2a and probed with the *EcoRI*-*SacI* fragment. Approximately 20 positives were obtained for each plate (Figure 4.11.a.). Replicas were made of each plate and grown overnight at 37°C. 50 colonies from areas surrounding the positive signals were picked, spotted onto agar plates in a grid system and reprobbed. From these 2 positives were obtained and minipreps performed on them.

The results of restriction digests with *SacI* and *EcoRI* indicated that both preps contained the insert but in the incorrect orientation (Figure 4.11.b.). *EcoRI* digestion is the diagnostic restriction for orientation. In the correct orientation three fragment sizes of 1.44, 2.25, and 6.01 Kb are seen representing as follows: *GPD* promoter and *DChE* gene fragment (0.65 Kb (Schena et al, 1991) + 0.790 Kb (from Figure 4.2) = 1.44 Kb); yeast 2 $\mu$  fragment (2.25 Kb Schena et al, 1991); remaining vector-gene fragment including *TRP1* and *pUC18* sequences (6.01 Kb). Insertion of the gene in the opposite orientation leads to relocation of the internal *EcoRI* site further downstream with respect to the *GPD* promoter which results in a larger promoter-*DChE* gene fragment of 2.13 Kb (0.65 Kb + 1.48 Kb (from Figure 4.2) = 2.13 Kb). The yeast 2 $\mu$  fragment of 2.25 Kb is unchanged and the remaining vector fragment becomes 5.32 Kb. A restriction map of pG3-*DChE* illustrating the sizes of the *EcoRI* fragments depending on which way the gene fragment is inserted is shown in Figure 4.12.

It can be seen in Figure 4.11.b. that the *EcoRI* digest gave the same pattern of three fragments for both preps: one of approximately 5 Kb (gene + vector) and two of approximately 2 Kb (one is the 2 $\mu$  fragment from the vector and the other the *GPD* promoter + *EcoRI* fragment of the gene in the incorrect orientation). There is some contamination of the positive clone preps with religated pG3 (visible in lanes 4 and 5).

Figure 4.11.a. Positive clones were identified by  $^{32}\text{P}$  labelled probing of lifts performed on *E. coli* transformed with unphosphatased pG3 ligations. A 1 in 10 dilution of pellets gave approximately 500 transformants per plate.

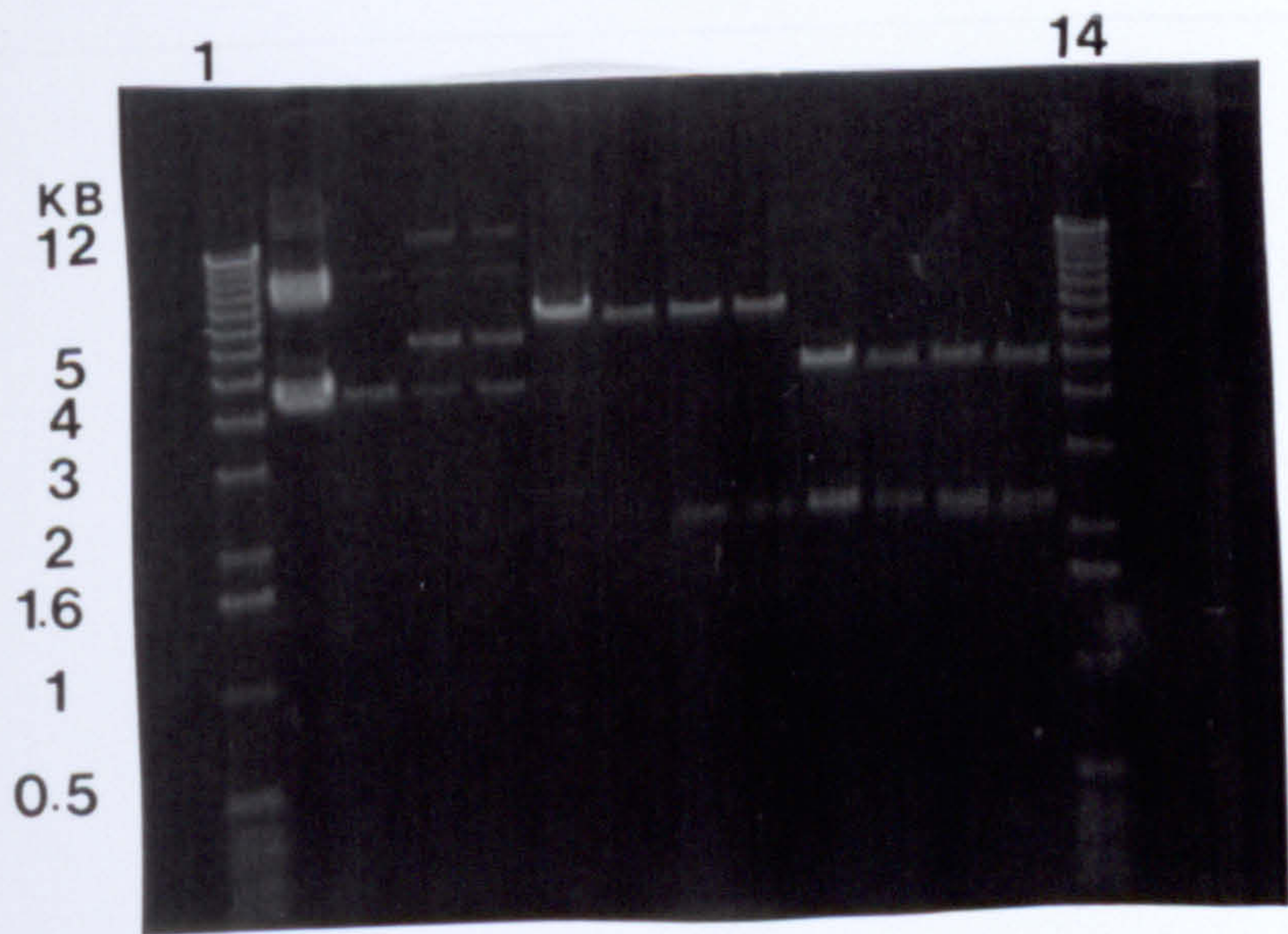
Figure 4.11.b. Positives were isolated by grid spotting and subsequent reprobing.

Lane 1 - 1 kb ladder  
2 - Uncut control pG3  
3 - Uncut negative control prep  
4 - Uncut positive clone 1  
5 - Uncut positive clone 2  
6 - *SacI* digest control pG3  
7 - *SacI* digest negative control prep  
8 - *SacI* digest positive clone 1  
9 - *SacI* digest positive clone 2  
10 - *EcoRI* digest control pG3  
11 - *EcoRI* digest negative control prep  
12 - *EcoRI* digest positive clone 1  
13 - *EcoRI* digest positive clone 2  
14 - 1 Kb ladder





A

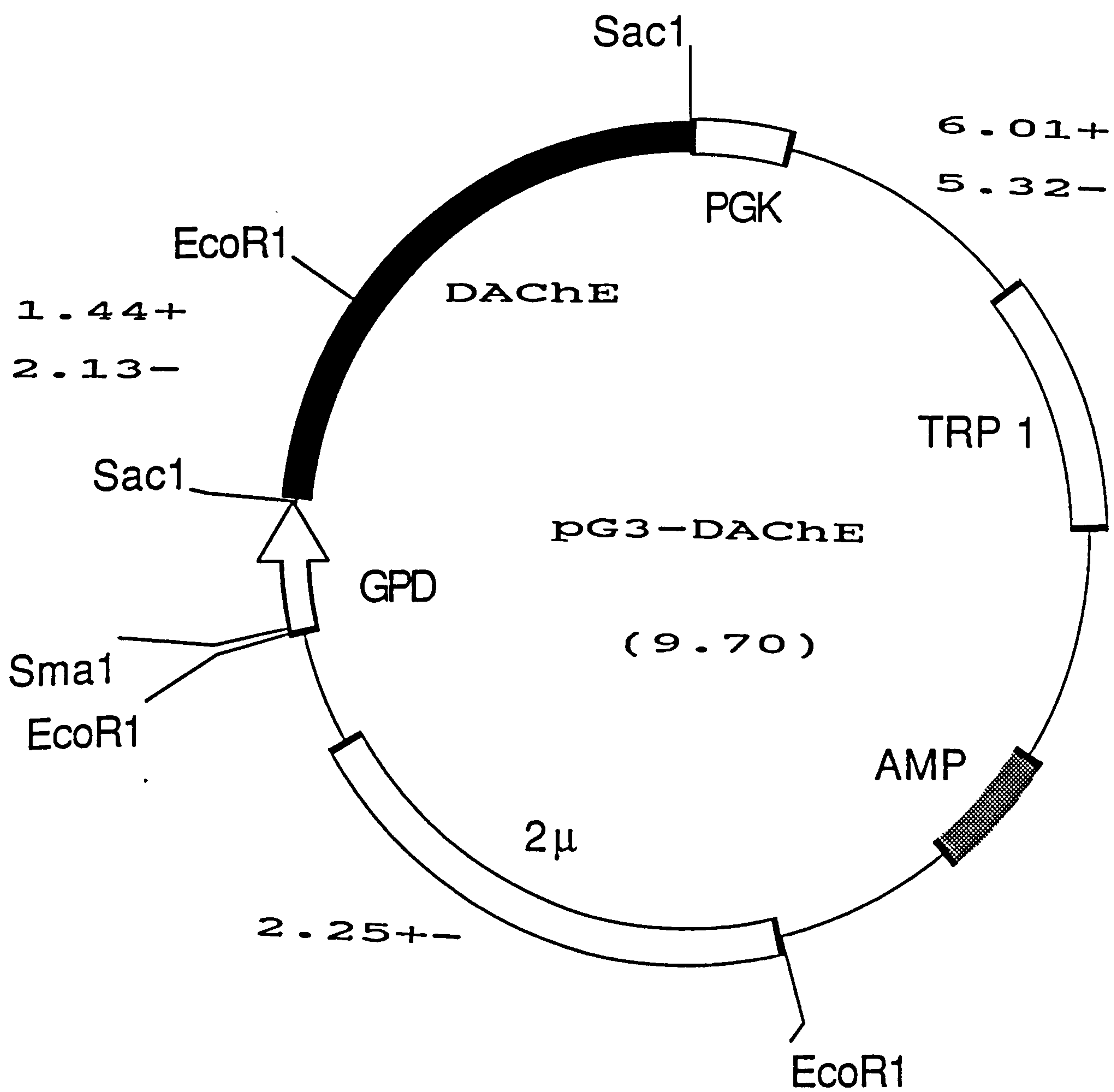


B



Figure 4.12. The *D. melanogaster* AChE gene under the *GPD* promoter in plasmid pG3. The exact sizes (Kb) of the individual *EcoRI* restriction fragments depending on which way the DACHe gene is inserted are illustrated: + = correct orientation; - = incorrect orientation. For full explanation see text.





To obtain a clone containing the gene in the correct orientation 484 colonies from plate 3a were spotted in a grid system on a large library agar plate and a colony lift performed. Probing of the filter revealed approximately 20 positives (Figure 4.13.a.). Of 10 clones 3 contained the gene in the correct orientation (clones 2, 5, and 6 Figure 4.13.b.). It can be seen in the digests of these clones that the *GPD* promoter + *EcoRI* fragment of the gene in the correct orientation is approximately 1.5 Kb. There is some contamination of religated pG3 in clones 3, 4, and 10.

#### 4.3.3. Subcloning into pPIC9

The 2.8 Kb gene fragment liberated by digestion of pKS-DACHE with *PvuI* followed by gel purification is shown in Figure 4.14.a. The double stranded oligonucleotide restricted with *PvuI*, purified by phenol:chloroform EtOH precipitation and then recovered by ultracentrifugation is shown in Figure 4.14.b. The gene fragment following ligation of the oligonucleotide and subsequent *XhoI* digestion and purification can be seen in Figure 4.14.c. Both bands are very faint. Transformation efficiencies following ligation of the gene fragment with Bluescript KS+ were high (>>1000 colonies per plate for 200 $\mu$ l *E. coli* pellet plated). White colonies were picked, restreaked to purity on X-gal, and minipreps performed. The results of 25 minipreps are shown in Figure 4.11.d. It can be seen that the ligations were unsuccessful. The white colonies proved to be false positives containing only Bluescript religated on itself. A direct ligation of the gene fragment into *XhoI* digested pPIC9 followed by colony lifts and probing using the <sup>32</sup>P labelled *EcoRI*-*SacI* probe was also unsuccessful.



Figure 4.13.a. All transformants from plate 3a (see text) were grid spotted and subsequently reprobbed.

Figure 4.13.b. *EcoRI* digests of isolated positive clones. The sizes (Kb) of the three diagnostic *EcoRI* fragments depending on which way the fragment is inserted are indicated: + = correct orientation; - = incorrect orientation.

Lane 1	- 1 Kb ladder
2	- Uncut control pG3
3	- Uncut negative control prep
4 to 13	- Uncut positive clones 1 to 10
14	- <i>EcoRI</i> control pG3
15	- <i>EcoRI</i> negative control prep
16 to 25	- <i>EcoRI</i> positive clones 1 to 10
26	- 1 Kb ladder



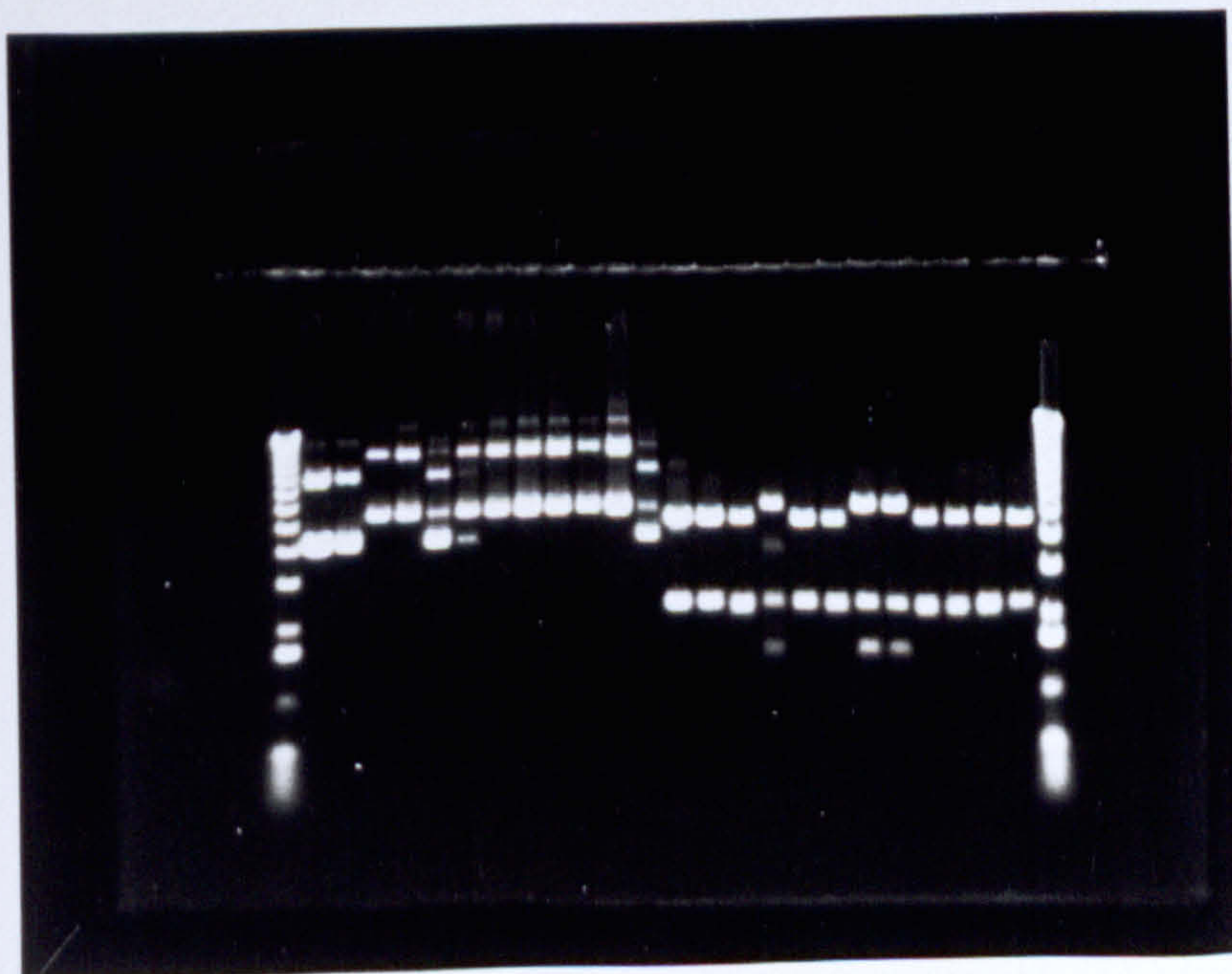


A

1

26

KB  
12  
4  
3  
2  
1



6.01+  
5.32-  
2.25+-  
2.13-  
1.44+

B



Figure 4.14.a. The *PvuI* DACH<sub>E</sub> gene fragment following digestion and gel purification.

Lane 1 - 1 Kb ladder  
2 - 2.8 Kb *PvuI* gene fragment

Figure 4.14.b. The *PvuI* restricted oligonucleotide was recovered by ultracentrifugation (2% w/v agarose).

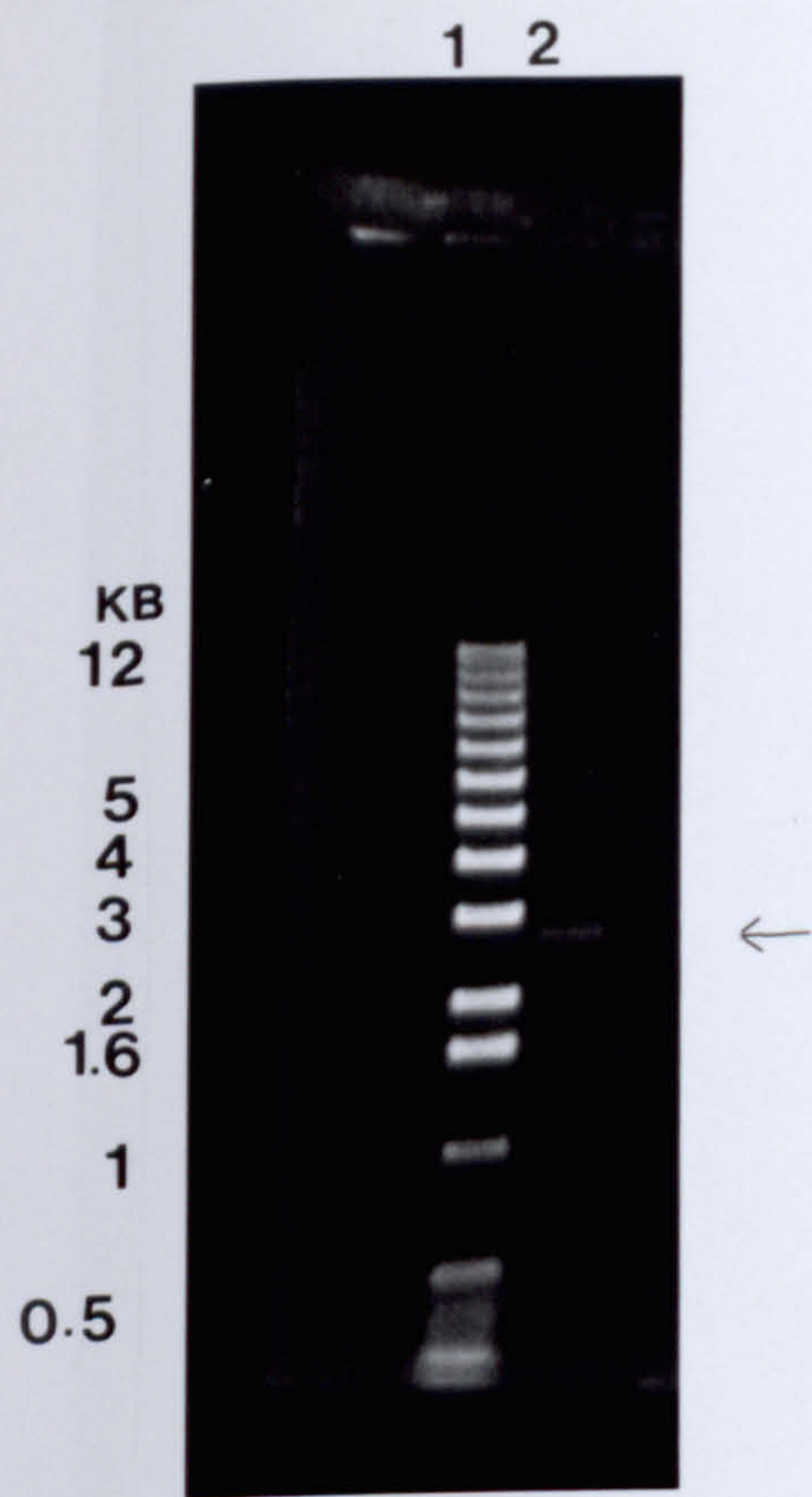
Figure 4.14.c. The oligonucleotide was ligated to the DACH<sub>E</sub> gene at *PvuI* sites and then restricted with *XhoI*. The DACH<sub>E</sub> gene fragment and ligated oligonucleotide runs slightly above the control indicating that ligation had been successful.

Lane 1 - 1 Kb ladder  
2 - DACH<sub>E</sub> gene fragment  
3 - DACH<sub>E</sub> gene fragment ligated to oligonucleotide

Figure 4.14.d. Minipreps of ligations into pKS<sup>+</sup> examined for the presence of inserts.

Lane 1 to 13	- Uncut miniprep plasmids
14	- Uncut pKS <sup>+</sup> control
15	- 1 Kb ladder
16 to 27	- Uncut miniprep plasmids

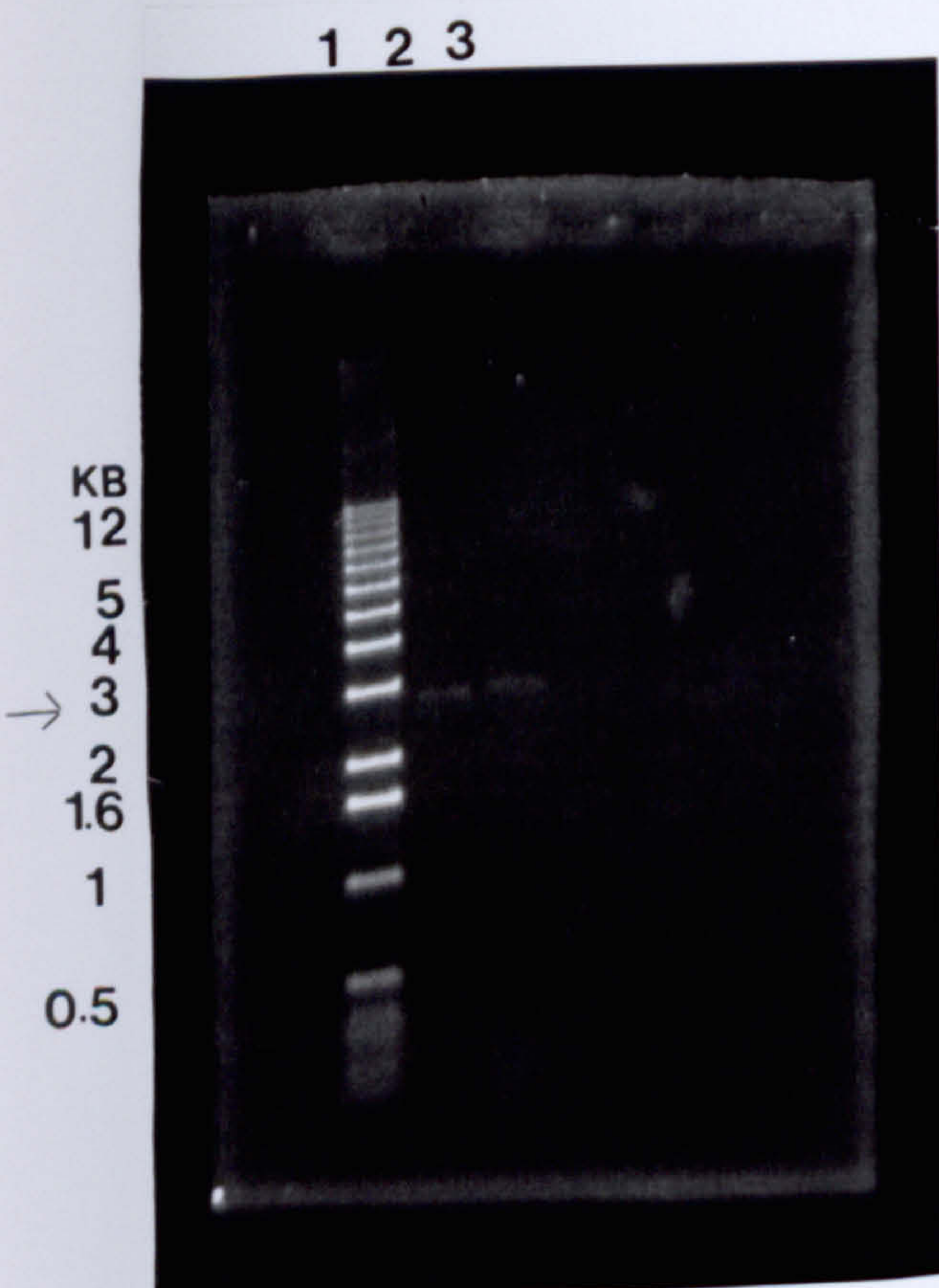




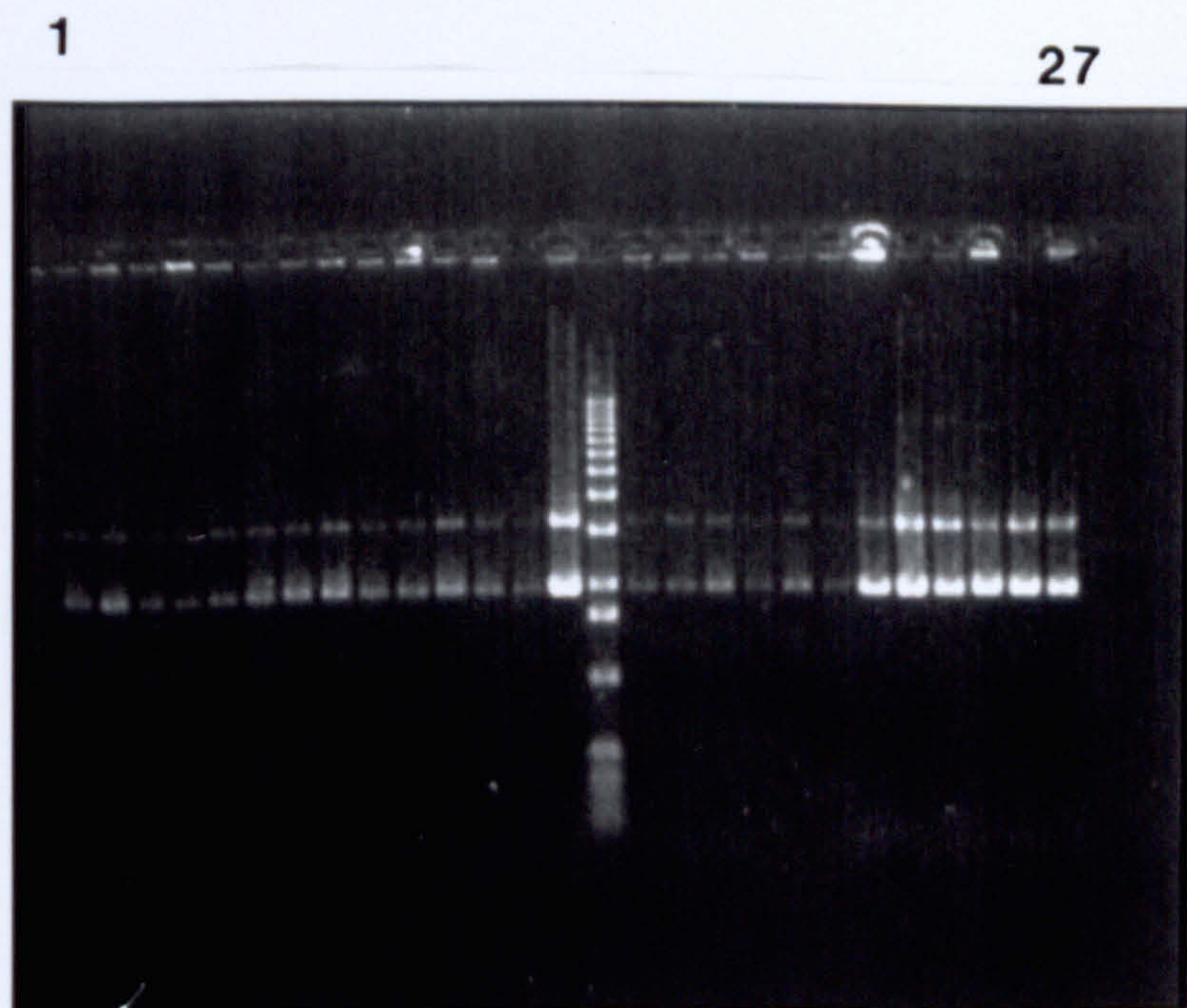
A



B



C



D



#### 4.4. Discussion

The *D. melanogaster* AChE gene was successfully subcloned into the modified yeast expression vector pG3 using *SacI* linkers. The orientation of the gene was confirmed by restriction digest with *EcoRI* (Figure 4.13.b). Assays for the expression of active AChE from the single copy galactose inducible vector pMB150 had indicated zero to very low levels of expression. The insertion of the gene under a powerful metabolic promoter (*GPD*) in a high copy number episomal plasmid offered a new possibility for expression. Plasmid p2UG offered a similar possibility with the advantage of controllable induction should the DACHÉ prove deleterious to cell growth.

The secretion vector pPIC9 offered other advantages (4.1). However, subcloning into these different expression vectors proved to be a difficult and lengthy process. There were several unsuccessful attempts to subclone the gene into p2UG and pPIC9 and the gene was only subcloned into pG3 in the correct orientation after an exhaustive screening procedure. Each vector posed different problems with regard to cloning options which were solved in theory by the strategy adopted. A difficulty with the yeast expression vectors available was the very limited number of restriction sites within the polylinker compared with *E. coli* vectors such as pUC19. Over the 2+ Kb of DACHÉ cDNA this meant that very often the gene shared sites in common with the polylinker making subcloning more difficult. For this reason it was decided to use the *BamHI* site in p2UG even though there are four *BamHI* sites within the DACHÉ cDNA. The *SacI* site available was thought inappropriate for cloning into p2UG because unlike pG3, p2UG does not contain a transcription terminator or polyadenylation site downstream of the polylinker. A *SacI* digest of the DACHÉ cDNA liberates a fragment cut only a short distance

downstream of the translation terminator and considerably upstream of the potential polyadenylation sites mentioned by Fournier et al (1989). In contrast cloning of the entire cDNA on a *SmaI-Hinc II* fragment with *BamHI* linkers includes these potential polyadenylation sites. Methylation of the endogenous *BamHI* sites within the gene made subcloning more problematic with regard to DNA losses through methylation steps and subsequent purification procedures.

Phosphatasing of the expression vectors was also a problem. There was no simple screening procedure available for the yeast vectors analogous with the blue/white screen for inserts available with pUC19 or Bluescript. Removal of the 5' phosphate groups from linearized vector termini prevents recircularization during ligation. It was found that phosphatasing of the vector pG3 with CIAP reduced the number of vector only transformants recovered by approximately 10 fold compared to unphosphatased controls (Table 4.9). Phosphatasing of vector p2UG with CIAP was found to reduce the number of vector only transformants but the transformation efficiency of DH5 $\alpha$  *E. coli* with p2UG proved to be extremely poor (Tables 4.7 and 4.8). Quite why p2UG transformed so poorly remains unclear, perhaps another bacterial strain would have been more suitable. All attempts to ligate the DACH $\epsilon$  gene into phosphatased vectors were unsuccessful. The reason remains unclear.

A 1.5 Kb fragment from an *EcoRI-SacI* digest of the DACH $\epsilon$  cDNA was used as a probe both with ECL and labelled with  $^{32}\text{P}$  to screen colony blots from unphosphatased vector/gene ligations. Probe concentration being too low was thought to be an initial problem with the ECL kit and increasing it did provide a possible positive in one of the p2UG ligations (4.3.1.). The low transformation efficiencies encountered with p2UG meant that a successful ligation was difficult to pick up. A typical frequency of insertion of



0.5% for example would require a transformant recovery of at least 200 colonies, at least double the actual numbers recovered (Table 4.8.).

Test transformations using pG3 had shown that this vector transformed well and direct ligation of the DAcHE cDNA on a SacI fragment was eventually successful. As previously mentioned pG3 contains a transcription terminator and polyadenylation signal from the PGK gene. The main problem throughout this subcloning was the loss of DNA encountered through the various steps involved in the strategy. This was eventually solved by subcloning through pUC19 which meant that as much DAcHE DNA as was needed could be obtained by cutting out on a simple SacI fragment from pUC 19. There was also a problem of contamination of the purified gene fragment with the similar length vector fragment. This was solved by additional digestion with KpnI (4.2.2.). The obvious importance of orientation also lengthened the procedures since isolation of positives was not simply a matter of cloning through to purity because additional cloning of a positive containing the gene in the incorrect orientation simply increases the number of unwanted positives. If the original ligation gave a possible 20 positives out of 500 then it was necessary to isolate all 20 individually in order to ascertain which, if any, contained an insert in the correct orientation. This was solved by picking all 500 colonies and regrowing in a grid system before probing (4.3.2.).

Subcloning into the secretion vector pPIC9 required the fusion of the  $\alpha$ -factor secretion signal in frame with the gene. A PvuI digestion of the DAcHE cDNA liberated a fragment minus the coding region for first 38 amino acids of the protein, a hydrophobically rich region that may have interfered with successful secretion. The recovery of the PvuI digested oligonucleotide by ultracentrifugation was successful (Figure 4.14.b.) and ligation of the

oligonucleotide to the *PvuI* digested gene fragment also appeared to be successful as shown in Figure 4.14.c. (the gene fragment with ligated oligonucleotide is slightly higher on the gel than the simple gene fragment). The problem of DNA loss encountered with the pG3 subcloning and the subsequent successful cloning through pUC19 suggested that a similar strategy be adopted. Furthermore, if orientation could be confirmed then there was the option of cutting the fragment out with two enzymes thereby simplifying the final ligation into the secretion vector - no vector self-ligation and all positives clones should contain an insert in the correct orientation. pUC19 could not be used because it did not offer the right combination of restriction options within the polylinker; Bluescript KS+ was used instead which gave the possibility of cloning into a *XhoI*-*NotI* site (the other three sites available within pPIC9 also occur within the DAcHE cDNA).

The subcloning into Bluescript was unfortunately not successful (4.3.3), a number of false white colonies being obtained (due probably to naturally occurring disruptions within the *lacZ* gene). An attempt to subclone directly into the *XhoI* site of pPIC9, rather than through Bluescript initially, also met with no success. Given the length of time involved in subcloning into pG3 it was decided to proceed with the analysis of pG3-DAcHE rather than continue with the pPIC9 subcloning.



## CHAPTER FIVE ANALYSIS OF DACH<sub>E</sub> EXPRESSION FROM CONSTRUCT pG3-DACH<sub>E</sub>

### 5.1. Introduction

The Sabine method of AChE detection (Sabine, 1955) originally developed for the routine testing of human erythrocyte cholinesterase titres is an extension of the method developed by Hestrin (Hestrin, 1949). The method is based on the reaction of acetylcholine with hydroxylamine to form acethydroxamic acid which forms a coloured complex with ferric ions that can be detected by absorbance at 540nm.

The Sabine assay was modified for use with the yeast expression system and was principally employed in the initial analysis of expression from pG3-DACH<sub>E</sub> to avoid the possibility that low levels of expression would remain undetected by the Ellman method because of cell wall interference (3.3.2). Use of the Sabine method also meant that disruption of cells with glass beads could be used for enzyme extraction with the advantage that low temperatures could be maintained throughout.

Biologically active DACH<sub>E</sub> was successfully expressed from construct pG3-DACH<sub>E</sub> in the *S. cerevisiae* strain BJ2168. This activity was found to be predominantly associated with the cell pellet. Accurate assessments of specific activity were therefore impossible when glass beading was used as the method of extraction because the presence of unbroken cells and portions of cell wall resulted in a heterogeneous suspension which made protein estimations unreliable. Extracts of protoplasts from exponentially growing cells were used to estimate activity in terms of specific activity.

DACHE expression from protoplasts was also analysed using the Ellman method to assess the feasibility of using the Ellman microtitre plate assay for inhibition studies. The aim was to establish that rates of activity of the recombinant DACHE could be successfully detected against the level of interference associated with protoplasts and that using protoplasts an expression system for insect AChE in *S. cerevisiae* could be employed in the testing of insecticides on AChE variants.

## 5.2. Materials and Methods

pG3-DACHE containing the gene in both the correct and incorrect orientations was electroporated into the protease deficient *S. cerevisiae* strain BJ2168 (2.7) to give the transformants BJ2168R and BJ2168W respectively. These strains together with cultures of untransformed BJ2168 were grown to the appropriate OD<sub>600</sub>, split and processed in triplicate, enzyme extracted using glass beads or lysed protoplasts, and assays for AChE conducted either by the method of Sabine (1955) or Ellman et al (1961) (2.22).

Initial assays using the Sabine method of crude extract supernatants and total cell extracts utilized glass beads as the method of extraction (2.22.2). Final resuspension was in 0.5ml 67mM phosphate buffer. Total cell extracts or supernatants (cell debris was removed by microfuging at 13000 rpm for 3 min and aspirating off the supernatant) were incubated with 0.5ml 8mM acetylcholineiodide (ACHI) and subsequently assayed (2.22.5).

The carbamate insecticide Bendiocarb (Bayer) (C<sub>11</sub>H<sub>13</sub>NO<sub>4</sub>, Mwt. 223.2) was used in a preliminary inhibition study at a final concentration of 10mM. It was added to wells of a microtitre plate containing protoplast extracts prior to assay by the Ellman method (2.22.4).



### 5.3. Results

Tabulated data together with calculations of specific activities +/- S.E.M. are given in Appendix 2.

#### 5.3.1. Preliminary Analysis of Expression using Minor Modifications of the Sabine Assay

Results obtained from initial attempts to assay for the presence of AChE in BJ2168R extracts by the method described by Sabine (1955) showed poor reproducibility. A series of preliminary experiments revealed the following were to blame:

1) The concentration of phosphate buffer in the assay is reported by Sabine as 0.67M. Preliminary assays for the presence of AChE (data not shown) which revealed an extremely low level of activity were severely hampered by precipitation of phosphate after the addition of  $\text{FeCl}_3$ .

2) Triton X-100 at a concentration of 1% v/v in crude extracts is routinely used in the assaying of insect AChE by the method of Ellman et al (Moores et al, 1988; Denholm et al, 1990; French-Constant and Bonning, 1989). A colloidal problem was encountered when Triton X-100 was used in the Sabine assay even at 0.1% v/v. The colloidal type interaction between cell protein, Triton, and  $\text{FeCl}_3$  could be substantially removed by centrifugation (13000 rpm, 15 min, MSE microfuge) but was not eliminated and gave rise to inconsistencies with the standard (data not shown).

3) Lowering of assay sensitivity by exposure of the ferric chloride solution to light was also a problem. A 10% w/v  $\text{FeCl}_3$  solution is described by Hestrin (1949) but Sabine reports (1955) the use of a 20% w/v solution.

4) Simple filtration through Whatman N° 1 paper following the addition of trichloroacetic acid (TCA) was also found to be inefficient at completely removing particulate material from crude yeast extracts which led to inconsistencies in spectrophotometer readings.

The published procedure was therefore modified to make the system suitable for the analysis of yeast extracts and achieve reproducibility:

1) The concentration of phosphate buffer in the assay was reduced 10 fold to 67mM. This effectively eliminated interference by precipitated phosphate in the assay procedure and was of a sufficient concentration to support enzyme activity.

2) Triton X-100 was omitted in the crude extracts and found to be unnecessary for activity. This solved the colloidal problem initially encountered.

3) FeCl<sub>3</sub> solutions were made up just prior to use and exposure to light was limited as much as was practicable. Final absorbances at 540nm obtained using a 10% w/v solution were found to be similar to those obtained with a 20% w/v solution (data not shown). Use of a 10% w/v solution was therefore adopted.

4) Centrifugation of samples (13000rpm, 10 min, MSE microfuge) after addition of TCA and filtration was found to improve the consistency of spectrophotometer readings (data not shown). Consistency was improved still further by omission of the filtration step and instead centrifuging immediately after the addition of TCA. A 'clean' aliquot could then be aspirated off for use in the assay.



### 5.3.2. A Qualitative Assessment of Activity Using Crude Yeast Extracts Assayed by the Sabine Method

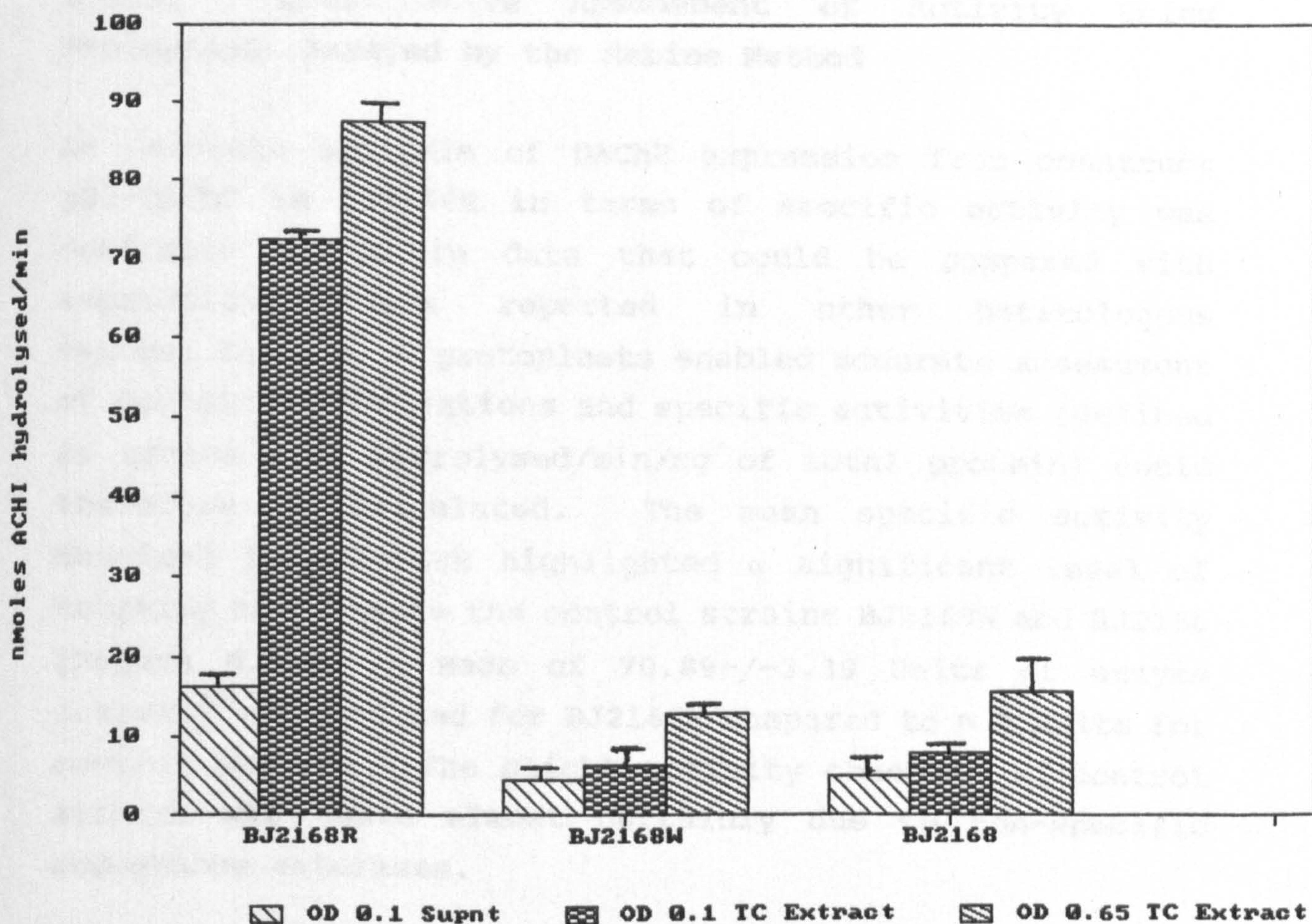
When crude extract supernatants from BJ2168R grown to an  $OD_{600}$  of 0.1 were assayed for DAcHE expression using the Sabine method there was some evidence of expression (Figure 5.1). It can be seen that there is little difference between the means of BJ2168W and BJ2168 but the presence of activity is clear from the difference between BJ2168R and the controls. The mean rate of substrate hydrolysis obtained from crude extract supernatants was found to be  $16.1 \pm 1.10$  nmoles/min. The absorbances at  $OD_{540}$  of the standards were slightly higher than the yeast extract controls indicating some additional hydrolysis of substrate by untransformed *S. cerevisiae*, probably by non-specific esterases. The data are interpreted in terms of nmoles of substrate hydrolysed per min. There was no attempt to quantify activity in relation to total protein at this stage.

When assays were performed on total cell extracts from exponentially growing cultures a higher level of activity was obtained from BJ2168R (Figure 5.1). A mean rate of hydrolysis of  $72.2 \pm 0.53$  nmoles of substrate hydrolysed/min was obtained from cultures grown to an  $OD_{600}$  of 0.1. Activity was therefore mainly associated with the cell pellet. Difficulties were encountered when an assessment in terms of total protein was attempted since the presence of unbroken cells and portions of cell wall in the suspension made protein readings unreliable. A quantitative assessment was achieved by the use of protoplasts (5.3.3).

An equally high recovery of DAcHE ( $87.2 \pm 1.50$  nmoles/min) was obtained from stationary phase cultures indicating that the enzyme continues to be expressed even in stationary phase (Figure 5.1). This may have implications for

Figure 5.1. Activity of transformed and untransformed *S. cerevisiae* cell extracts (glass bead method) from exponential and stationary phase cultures. Activity is expressed in terms of nmoles ACHI hydrolysed/min. 'Supt': supernatant fraction of total cell extract; 'TC Extract': total cell extract.







possible large scale production since biomass is significantly increased if cells can be harvested at stationary phase. A quantitative assessment of activity was not undertaken, however, due to the fact that stationary phase cells are extremely resistant to protoplasting.

#### **5.3.3. Quantitative Assessment of Activity Using Protoplasts Assayed by the Sabine Method**

An accurate analysis of DACH<sub>E</sub> expression from construct pG3-DACH<sub>E</sub> in BJ2168R in terms of specific activity was desirable to obtain data that could be compared with expression levels reported in other heterologous systems. The use of protoplasts enabled accurate assessment of protein concentrations and specific activities (defined as nmoles ACHI hydrolysed/min/mg of total protein) could therefore be calculated. The mean specific activity obtained for BJ2168R highlighted a significant level of activity compared to the control strains BJ2168W and BJ2168 (Figure 5.2). A mean of 70.89+/-3.39 Units of enzyme activity was obtained for BJ2168R compared to  $\approx 3$  Units for control strains. The slight activity observed in control strains was again almost certainly due to non-specific endogenous esterases.

#### **5.3.4. Quantitative Assessment of Activity Using Protoplasts and the Ellman Method**

Once the expressed DACH<sub>E</sub> had been detected by the Sabine method it was desirable for the long term aims of the project to examine the feasibility of using the Ellman method to analyse activity from lysed protoplasts of BJ2168R using a microtitre plate reader. Enzymatic digestion of the cell wall had already been shown to significantly reduce the level of interference (3.3.2.d) and Figure 5.3 shows that it was possible to detect a rate



Figure 5.2. Quantification of activity levels of DACH<sub>E</sub> extracted from *S. cerevisiae* protoplasts (Sabine method).

Units are defined in terms of nmoles ACHI hydrolysed/min/mg protein.

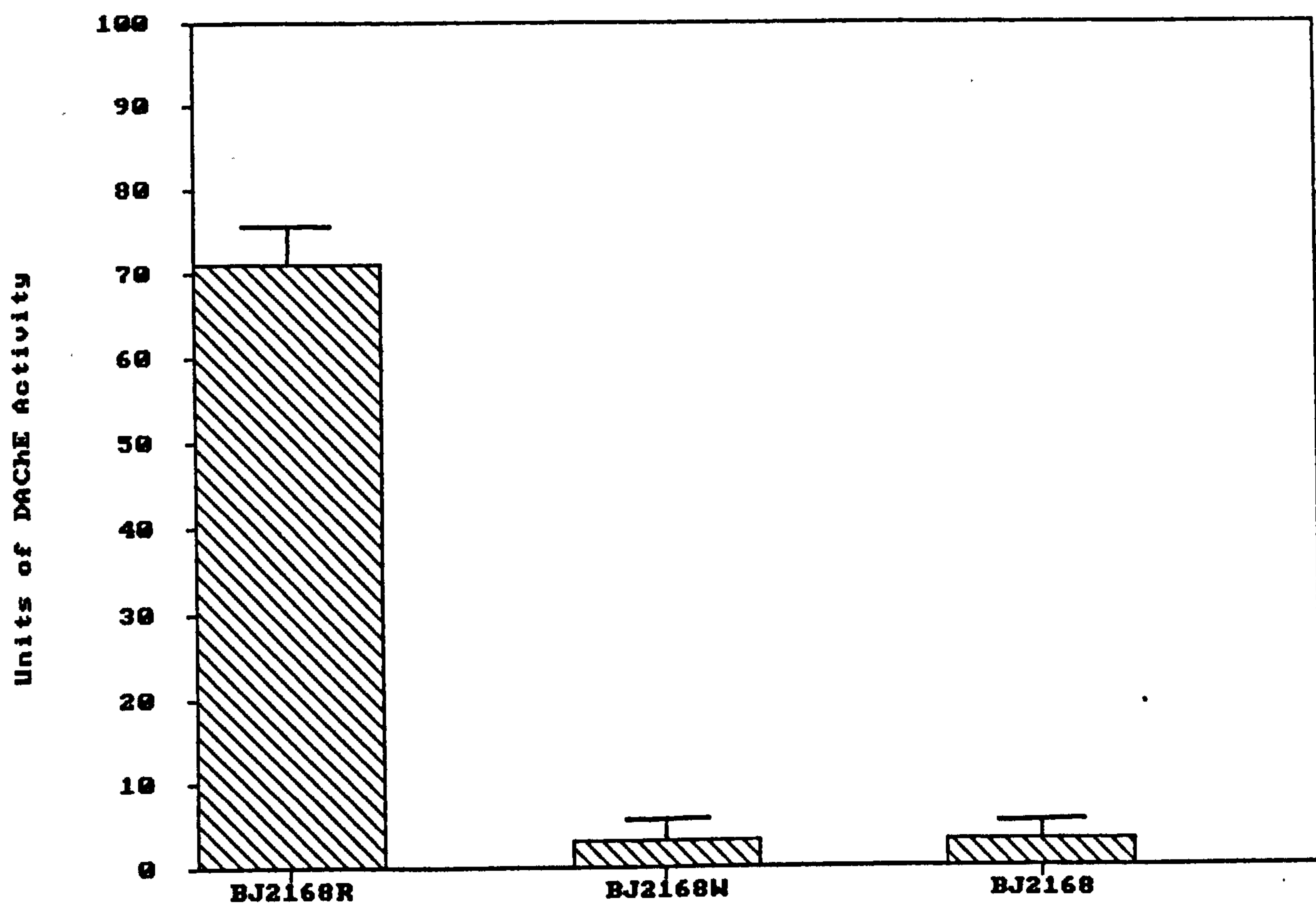
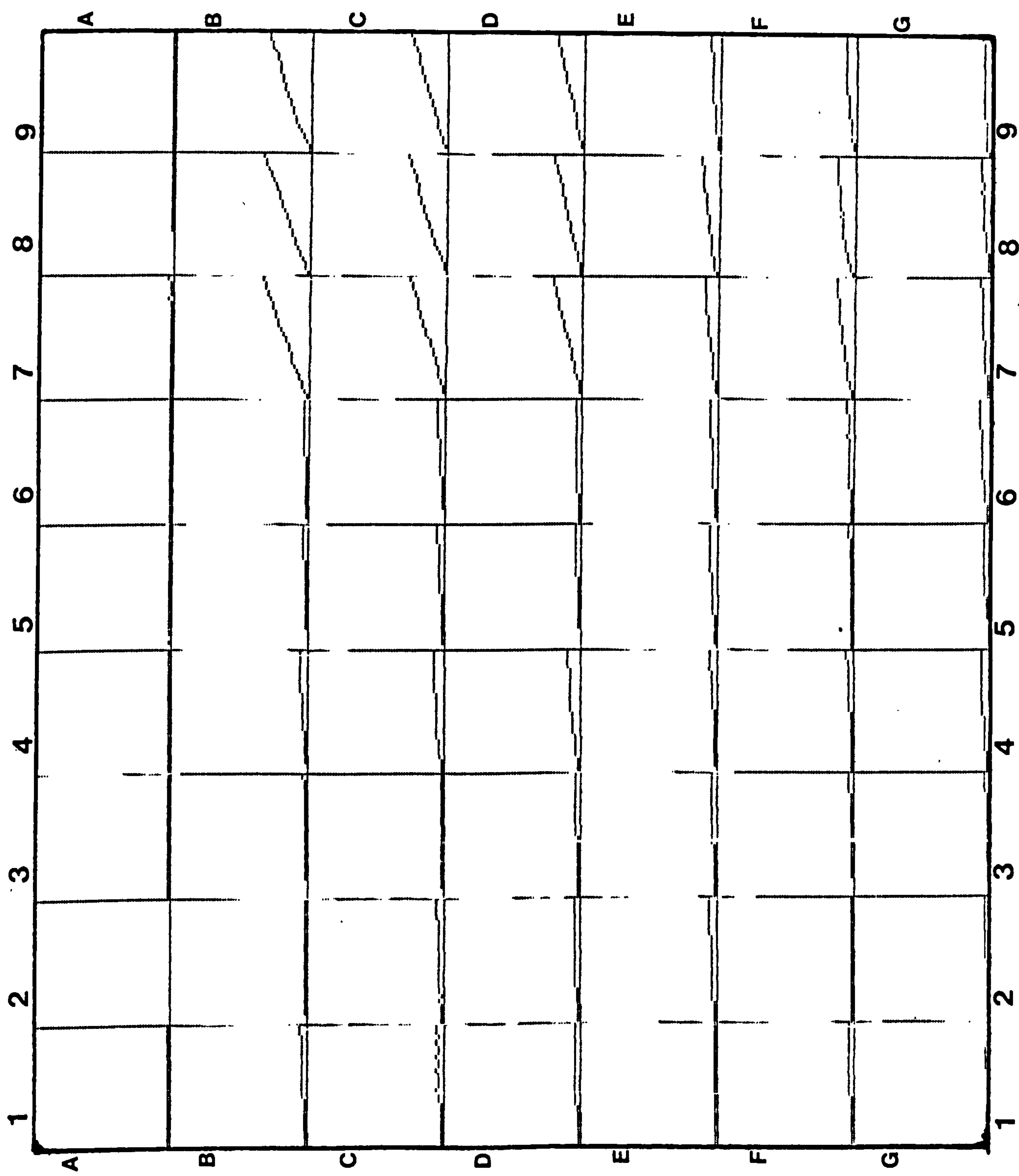




Figure 5.3. The kinetics of active DACHe from *S. cerevisiae* protoplasts measured by the Ellman assay. The effect of the insecticide Bendiocarb on DACHe activity is illustrated.

Group	Well Co-ordinates
BJ2168 Uninhibited	B1 - B3 C1 - C3 D1 - D3
BJ2168 Inhibited	E1 - E3 F1 - F3 G1 - G3
BJ2168W Uninhibited	B4 - B6 C4 - C6 D4 - D6
BJ2168W Inhibited	E4 - E6 F4 - F6 G4 - G6
BJ2168R Uninhibited	B7 - B9 C7 - C9 D7 - D9
BJ2168R Inhibited	E7 - E9 F7 - F9 G7 - G9
Blank	A1 - A9





of substrate hydrolysis by the recombinant DACH<sub>E</sub> above the level of interference associated with protoplasts. It can be seen that a linear increase in product (yellow anion 5-thio-2-nitro-benzoic acid) formation occurs in wells containing the recombinant gene in the correct orientation (BJ2168R). In contrast the formation of the yellow anion in control wells is both slow and erratic. Using the 'zoom' facility of the plate reader to examine the data of individual wells in greater detail the nature of the anion formation by means of typical enzyme hydrolysis of ATCHI and by cell wall interference can be seen (Figure 5.4). The kinetics are quite different. BJ2168R shows the typical linear kinetics characteristic of enzyme hydrolysis whereas the formation of the anion in the well containing BJ2168W is extremely erratic, providing further evidence of its non-enzymatic nature.

The extinction coefficient of the yellow anion is known (Ellman *et al*, 1961). Rates of change in absorbance can be therefore be converted to absolute units:

$$\text{rate (moles/l/min)} = \text{change in absorbance per min} / 1.36 \times 10^4$$

Mean specific activities in terms of 1 Unit of activity equal to 1 nmole of ATCHI hydrolysed/min/mg total protein and corrected for the blank are shown in Figure 5.5. 27.32+/-4.49 Units of activity were calculated for BJ2168R. Approximately 8 Units of activity are recorded for control groups and approximately 65% of this would be due to cell wall interference rather than non-specific endogenous activity. This conclusion is supported by the data from assays conducted using the Sabine method. Approximately 3 Units of activity were calculated as being associated with endogenous activity (Figure 5.2).

Non-specific activity is indistinguishable from interference due to remaining cell wall when the Ellman

Figure 5.4. The exponential formation of products due to enzyme hydrolysis of ATCHI and cell wall interference compared.

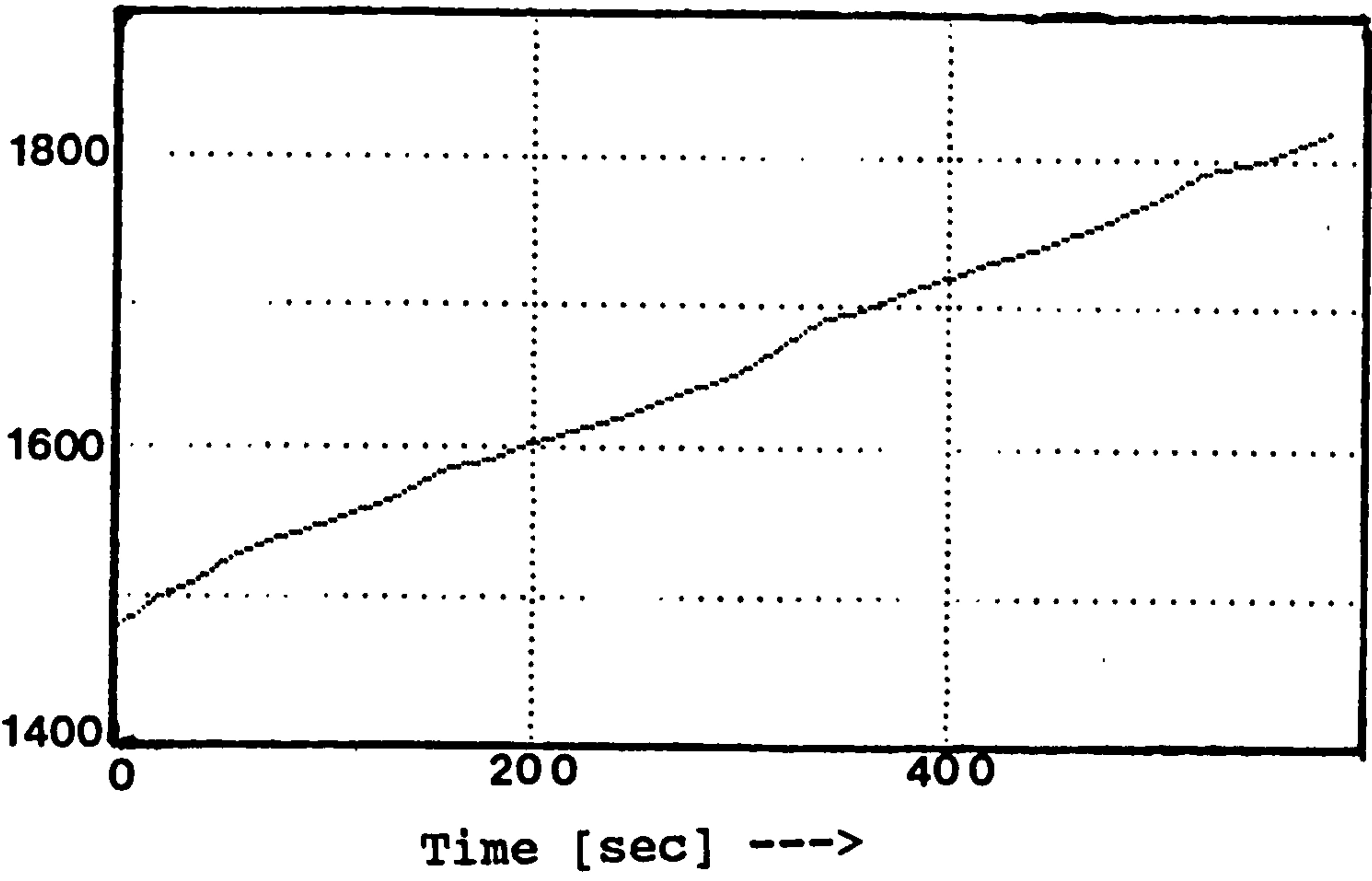
Well B7 Contained Lysed Protoplasts of BJ2168R.

Well B6 Contained Lysed Protoplasts of BJ2168W.

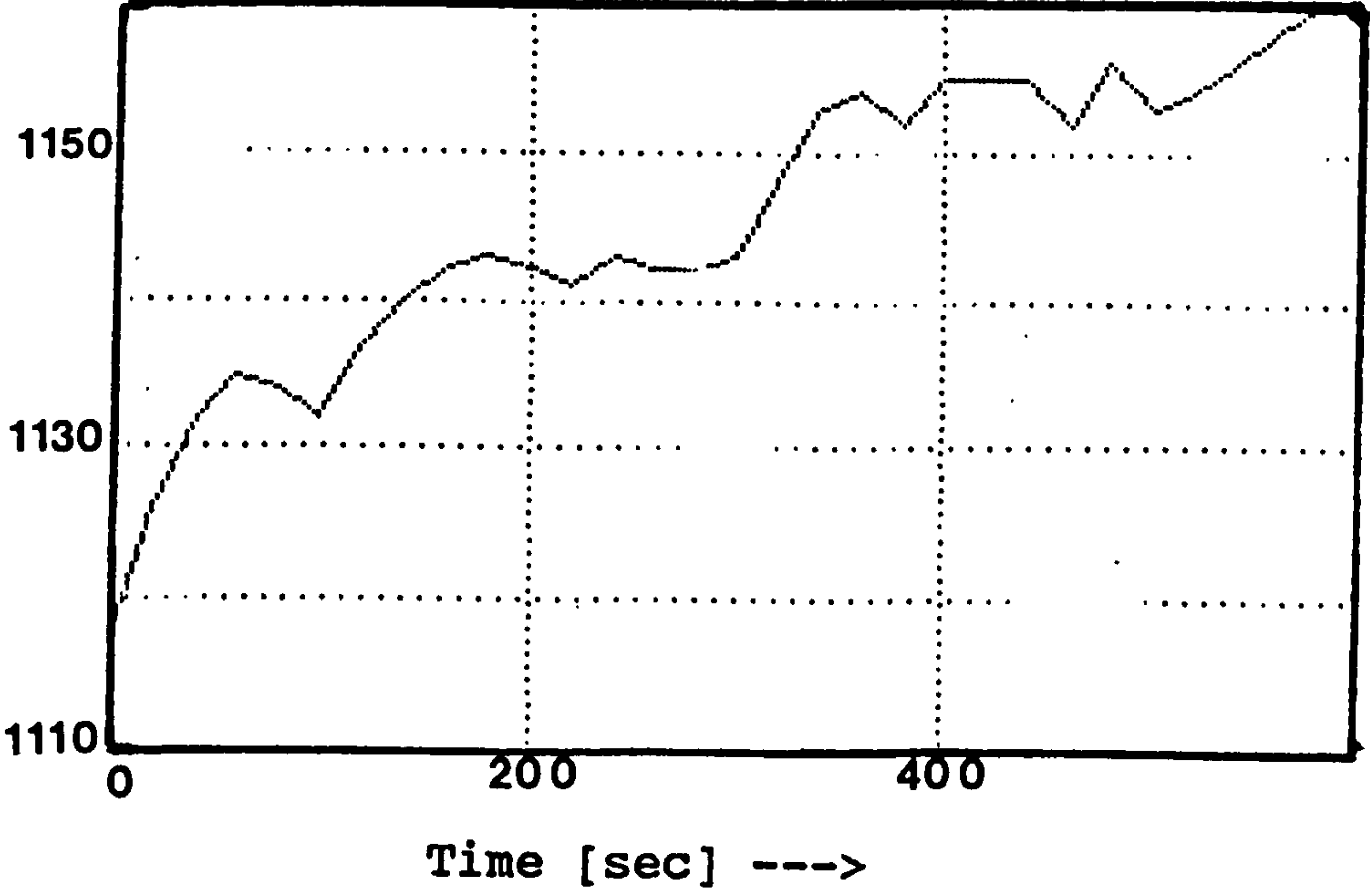


Well Position: B-7

Absorbance [mOD]



Absorbance [mOD]



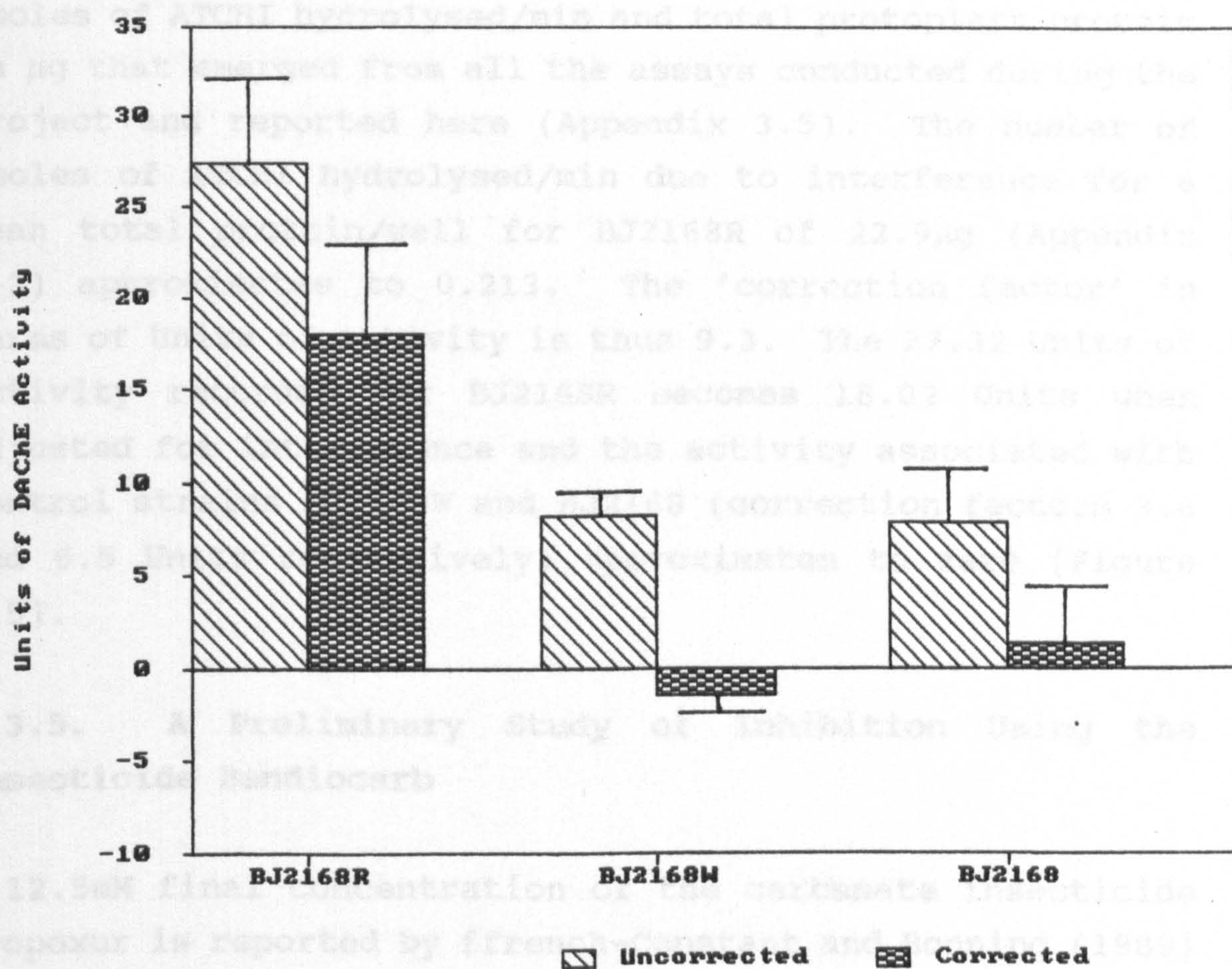
Well Position: B-6

Figure 5.5. Quantification of activity levels of DAcHE extracted from *S. cerevisiae* protoplasts (Ellman method). Controls show levels of activity derived mainly from cell wall interference. Activity levels are shown uncorrected and corrected for interference and endogenous enzyme activity (see text).

Units are defined in terms of nmoles ATCHI hydrolysed/min/mg protein.



method of detection is used with *S. cerevisiae* protoplasts. The total figure is desirable when corrections for this interference are made since it takes account of both forward and reverse activities and the slight activity observed in control strains and gives an accurate assessment of activity due to the expressed DACHE in BJ2168R. A 'correction factor' for cell wall interference can be approximated based on the straight line relationship between interference in terms of



5.3.5. A Preliminary Study of Inhibition Using the Insecticide Dieldrin. The insecticide Dieldrin was tested at a 10mM final concentration using purified AChE from *Torpedo marmorata* (Sigma). 0.019 Units of purified *Torpedo* AChE were completely inhibited by a final concentration of Dieldrin of 10mM (Figure 5.6).

The lower half of the microtitre plate result in Figure 5.3 shows the partial inhibition of the recombinant DACHE



method of detection is used with *S. cerevisiae* protoplasts. The total figure is desirable when corrections for this interference are made since it takes account of both interference and the slight activity observed in control strains and gives an accurate assessment of activity due to the expressed DAcHE in BJ2168R. A 'correction factor' for cell wall interference can be approximated based on the straight line relationship between interference in terms of nmoles of ATCHI hydrolysed/min and total protoplast protein in  $\mu\text{g}$  that emerged from all the assays conducted during the project and reported here (Appendix 3.5). The number of nmoles of ATCHI hydrolysed/min due to interference for a mean total protein/well for BJ2168R of  $22.9\mu\text{g}$  (Appendix 2.3) approximates to 0.213. The 'correction factor' in terms of Units of activity is thus 9.3. The 27.32 Units of activity recorded for BJ2168R becomes 18.02 Units when adjusted for interference and the activity associated with control strains BJ2168W and BJ2168 (correction factors 9.6 and 6.5 Units respectively) approximates to zero (Figure 5.5).

#### 5.3.5. A Preliminary Study of Inhibition Using the Insecticide Bendiocarb

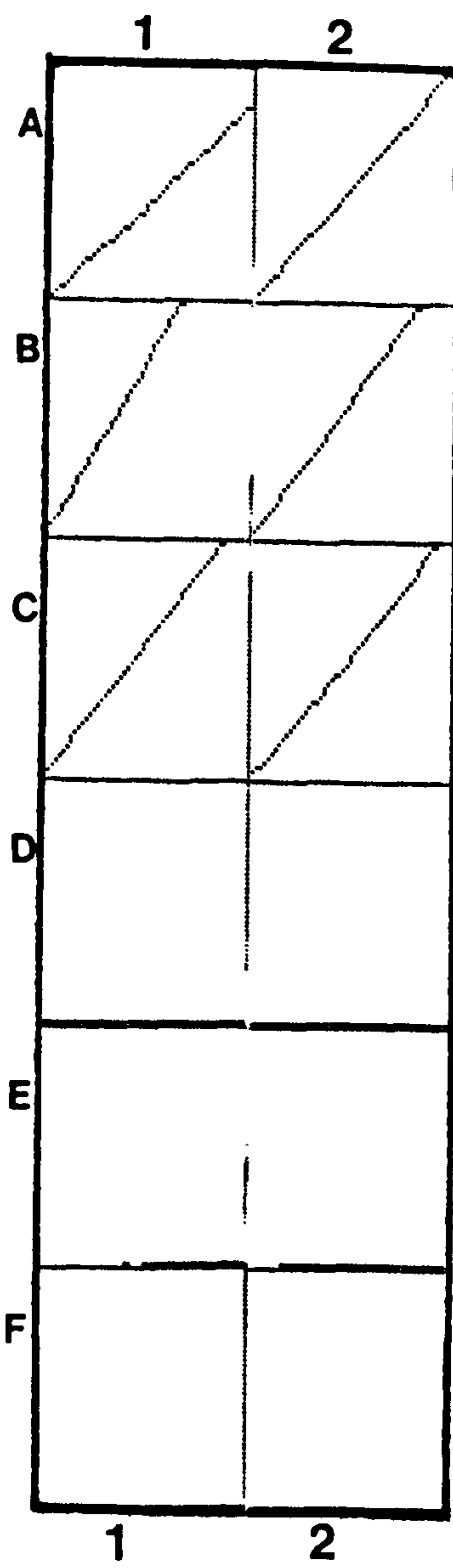
A 12.5mM final concentration of the carbamate insecticide Propoxur is reported by French-Constant and Bonning (1989) as sufficient to completely inhibit AChE in crude extracts from individual homozygous susceptible *An. albimanus* and *Cx. pipiens* when assayed by the method of Ellman et al on a microtitre plate reader. The insecticide Bendiocarb was tested at a 10mM final concentration using purified AChE from *Torpedo marmorata* (Sigma). 0.019 Units of purified *Torpedo* AChE were completely inhibited by a final concentration of Bendiocarb of 10mM (Figure 5.6).

The lower half of the microtitre plate readout in Figure 5.3 shows the partial inhibition of the recombinant DAcHE



Figure 5.6. The effect of the insecticide Bendiocarb on purified *Torpedo* AChE.

Group	Well Co-ordinates
<i>Torpedo</i> AChE Uninhibited	A1 - A2 B1 - B2 C1 - C2
<i>Torpedo</i> AChE Inhibited	D1 - D2 E1 - E2 F1 - F2





by a final 10mM concentration of Bendiocarb. The mean percentage inhibition (expressed as a percentage of uninhibited activity) was found to be  $50.9\% \pm 3.52$  for BJ2168R compared to  $86.9\% \pm 3.88$  and  $72.4\% \pm 7.90$  for strains BJ2168W and BJ2168 respectively (Figure 5.7). A mean figure for the control strains of 79.65% correlates well with an estimation of  $\approx 30\%$  non-specific activity, the bulk of the remainder being due to cell wall interference and therefore not subject to inhibition by insecticide.

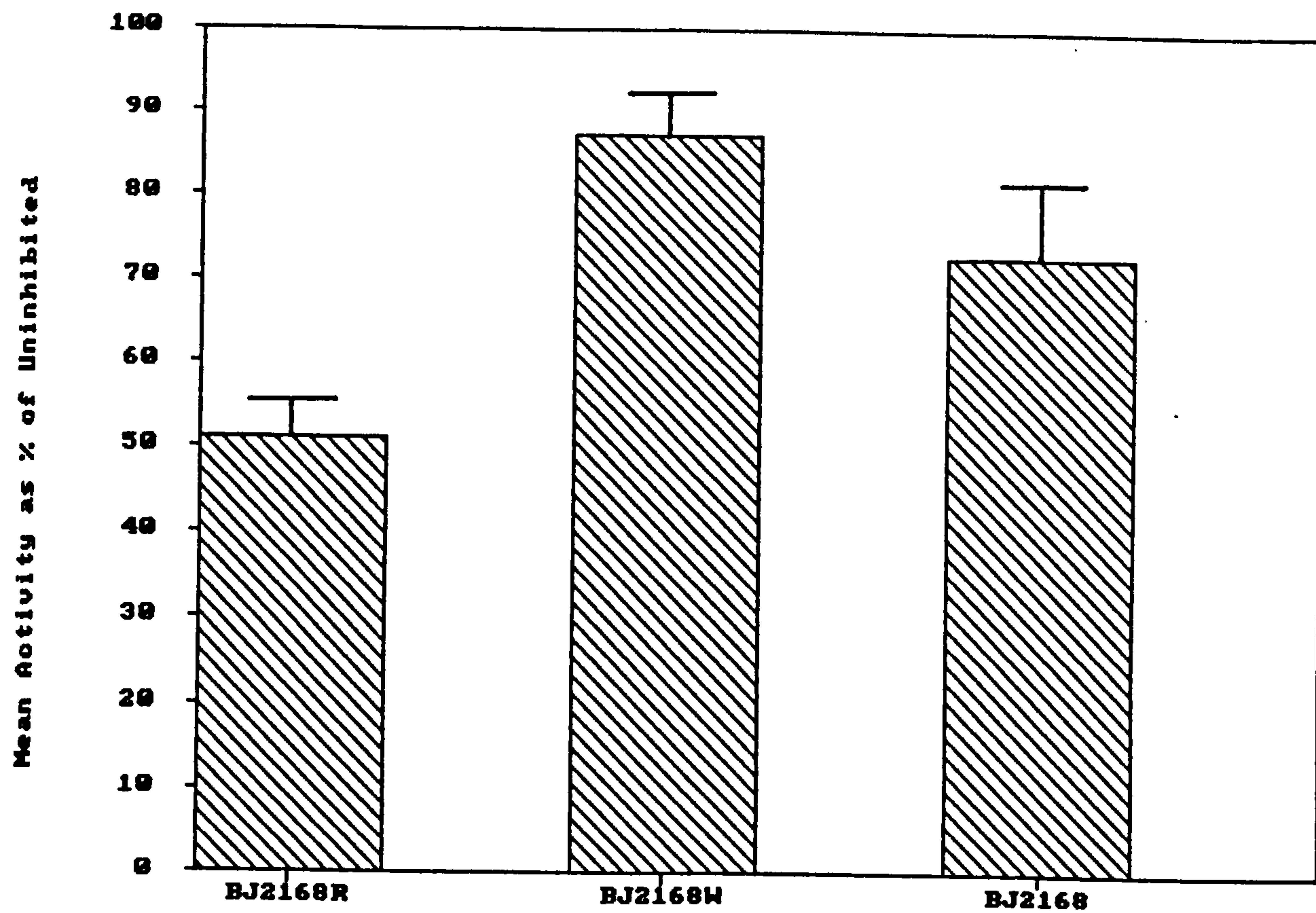
#### 5.4. Discussion

Biologically active AChE expressed from construct pG3-DACHE in *S. cerevisiae* strain BJ2168R was detected by both the Sabine and the Ellman assays. That the recombinant enzyme could be detected against the level of interference associated with protoplasts and the kinetics examined using a microtitre plate reader was of the utmost significance for the long term aims of the project. Partial inhibition of the recombinant AChE was achieved using the carbamate insecticide Bendiocarb. At a final concentration of 10mM the expressed DACHE was inhibited by only  $\approx 50\%$  compared to the 100% inhibition seen with the purified *Torpedo* enzyme. This would have been due to the 'mopping up' of the inhibitor by other proteins in the crude protoplast extract. The results demonstrate the feasibility of using a yeast expression system for the investigation of insecticide resistant variants of insect AChE.

Activity was found to be mainly associated with the pellet when the enzyme was extracted by breaking cells open with glass beads and subsequently assayed by the Sabine method (Figure 5.1). Unfortunately accurate comparative measurements of DACHE activity in terms of specific activity were impossible because the presence of unbroken cells and portions of cell wall created a heterogeneous suspension making protein estimates unreliable. Specific

Figure 5.7. The effect of the insecticide Bendiocarb on DACH<sub>E</sub> from BJ2168R compared to controls. The mean activity in the presence of inhibitor is expressed as a percentage of uninhibited levels.





activities obtained using protoplasts and the Sabine method gave a mean specific activity of  $70.89 \pm 3.39$  nmoles of substrate hydrolysed/min/mg of protein for transformants containing the gene in the correct orientation.

A quantitative comparison between the two methods of extraction used is not possible due to the absence of total protein data for the glass bead extractions. Levels of activity recorded for enzyme extracted from protoplasts and enzyme extracted using glass beads from approximately the same number of cells would seem to suggest that the latter method led to higher levels of activity (Appendices 2.1 and 2.2). This observation is further supported by estimations of cell lysis: resuspension of protoplasts in 100mM phosphate buffer should achieve  $\approx 80\%$  lysis in contrast to the 10-20% of cells typically disrupted by glass beads (data not shown).

The observation led to speculation as to the cellular location of the recombinant DACH<sub>E</sub> and whether this had a bearing on the difference seen between activity levels obtained by the two extraction procedures (6.3.5).

The level of interference in the Ellman assay created when yeast cells are disrupted with glass beads (3.3.1) meant that any investigation of its use for the long term aims of the project was limited to protoplasts. An activity level of  $27.32 \pm 4.49$  Units (18.02 Units when corrected for interference) was obtained (Figure 5.5). This was a much lower level of activity than the 70.89 Units calculated from assays using the Sabine method. Initially it was thought that expression of the DACH<sub>E</sub> might have been deleterious to cell growth and that the lower level of activity recorded was reflected in a reduction of plasmid copy number. Plasmid copy number measurements (6.3.1) showed that this was not the case. Subsequently it was discovered that the use of stationary phase cultures for



experimental inoculums had been the cause. Cultures for the earlier Sabine experiments had been inoculated using mid-exponential cultures.

Rosenberry et al (1972) describe a specific activity (when recalculated in terms of standard enzyme units) of AChE from *Electrophorus electricus* following toluene extraction and ammonium sulphate precipitation of  $\approx 520$  Units. The extraction and precipitation purification is assessed as a 20-30 fold improvement in specific activity which gives an approximate value of 20 Units for fresh tissue homogenate. The average specific activity for the crude preparation of recombinant DACHe from yeast was calculated at  $\approx 0.07$  Units (approximately 250-300 fold lower). Velan et al (1991) report a specific activity of human AChE from transfected 293 cells (human embryonal kidney cells) of 1500 nmoles substrate hydrolysed/min for approximately  $5 \times 10^6$  cells. This again is considerably higher ( $\approx 500\times$ ) than the activity of  $\approx 50$  nmoles/min obtained from approximately  $1 \times 10^8$  cells of BJ2168R (50ml at an  $OD_{600}$  of 0.1) as measured by the Sabine assay. A detailed discussion of relative expression levels of AChE in *P. pastoris*, *E. coli*, baculovirus systems, *Xenopus* oocytes, and mammalian cells, compared to the results of this study is given in 8.1

It is clear that the specific activity obtained from construct pG3-DACHe in *S. cerevisiae* was low. When human AChE was expressed in *E. coli* (Fischer et al, 1993) it was initially found to be embedded in inclusion bodies and therefore inactive. Refolding of the *E. coli* enzyme *in vitro* only resulted in a low level of activity similar to that seen here with the yeast enzyme (0.28 Units and 0.07 Units respectively). Replacement of the unpaired Cys<sup>580</sup> residue (thought to be involved in the formation of the dimer (Velan et al, 1991b)) with serine increased the refolding efficiency 40 fold with a concomitant increase in specific activity to 46.10 Units (Fischer et al, 1993;

1995). The *Drosophila* AChE contains two potential sites of refolding problems: Cys<sup>615</sup> (analogous to the human AChE Cys<sup>580</sup>) and an additional unpaired cysteine residue at position 328 (Mutero and Fournier, 1992). SDS-PAGE analysis was later used (6.3.4) to investigate the possibility that large amounts of the expressed DACH were inactive.

The relatively low level of activity may also have been the result of an adverse effect on cellular metabolism, caused by expression of the enzyme, resulting in the selection of cells carrying fewer copies of the plasmid. Plasmid copy number analysis (6.3.1) was required to determine whether or not this was the case and whether expression levels might be increased by modifying the construct, for example by introducing an inducible promoter.

Characterization of the expression from construct pG3-DACH at both the RNA and protein level was desirable in terms of further authenticating the yeast AChE and to assess the possibilities for increasing the levels of activity obtained. Information regarding the recombinant AChE's cellular location would also have implications for any prospective purification procedure.



## CHAPTER SIX CHARACTERISATION OF DACE EXPRESSION FROM CONSTRUCT pG3-DACE

### 6.1. Introduction

The intracellular expression of AChE might be expected to have an adverse effect on cellular metabolism, resulting in the selection of cells carrying fewer copies of the expression plasmid. The plasmid copy number of strain BJ2168R was therefore measured and compared with that of strain BJ2168W to ascertain whether expression levels could be increased by the attachment of an inducible promoter.

A low level of transcription or an unstable mRNA might also have been responsible for the relatively low specific activity observed (5.4). Both the transcript size and the quantities of the DACE mRNA produced from construct pG3-DACE in *S. cerevisiae* at four different stages of the growth curve were therefore assessed by Northern blotting.

Inhibition of the expressed DACE using the cholinesterase inhibitor PMSF was examined to both obtain a quantitative assessment of the amount of active enzyme present and to compare its inhibition profile with that of a purified AChE from *Torpedo*. These studies were further supported by the use of SDS-PAGE to analyse crude protoplast extracts from BJ2168R and BJ2168W to assess the amount of total DACE protein. The aim was an approximate calculation of the amount of active DACE as a percentage of total DACE protein expressed which would indicate whether specific activity was likely to be elevated by disaggregation and renaturation of the heterologous DACE.

Since it was possible that the DACE protein may not be processed in yeast in the same manner as it is processed in *Drosophila* and that it may undergo extensive glycosylation

leading to retardation in SDS-PAGE gels BJ2168R and BJ2168W cells were grown in the presence of tunicamycin (a known inhibitor of glycosylation) and samples subjected to SDS-PAGE analysis.

Analysis of enzyme extracts using cell breakage with glass beads had demonstrated that activity was associated with the cell pellet (5.3.2). This activity was found in pellets spun down in a microfuge at 13000 rpm for 3 minutes, indicating that the enzyme was likely to be associated with the cell membrane/cell wall rather than with microsomal or soluble fractions. Activity levels associated with the membrane fraction of protoplasts were assessed and compared with levels associated with whole protoplast extracts. The specific activity of the membrane fraction was found to be  $\approx 4$  fold higher and thus the membrane fraction was also analysed by SDS-PAGE. Assays were subsequently performed using osmotically stabilized BJ2168R protoplasts to assess whether the recombinant enzyme was present on the inside or the outside of the cell membrane.

The qualitative observation that activity levels were higher when enzyme was extracted using glass beads rather than from protoplasts (5.4) was further investigated by examining certain aspects of the protoplasting procedure to determine which factor or factors might be responsible.

## 6.2. Materials and Methods

*S. cerevisiae* strains BJ2168R, BJ2168W, and BJ2168 were grown, harvested, and protoplasted as detailed in 2.22.1 and 2.22.3. Unless detailed otherwise mid exponential cultures grown overnight to an OD<sub>600</sub> of 0.1 were used.

Plasmid copy numbers were estimated by digesting 5 $\mu$ g yeast genomic DNA (2.9) from each strain, running on a 0.8% w/v



agarose gel (2.12), Southern blotting (2.18) and probing (2.21) with pDCD112 (Figure 6.1). Densitometry tracings of the bands on the autoradiograph were used to calculate the number of plasmids per cell in relation to the number of copies of ribosomal RNA genes (Appendix 3.1).

Total RNA was extracted from cultures of BJ2168R at four different stages of the growth curve (Table 6.2) using Qiagen columns (2.26.b). Total RNA extracted from BJ2168W cultures at late exponential phase was used as a control.

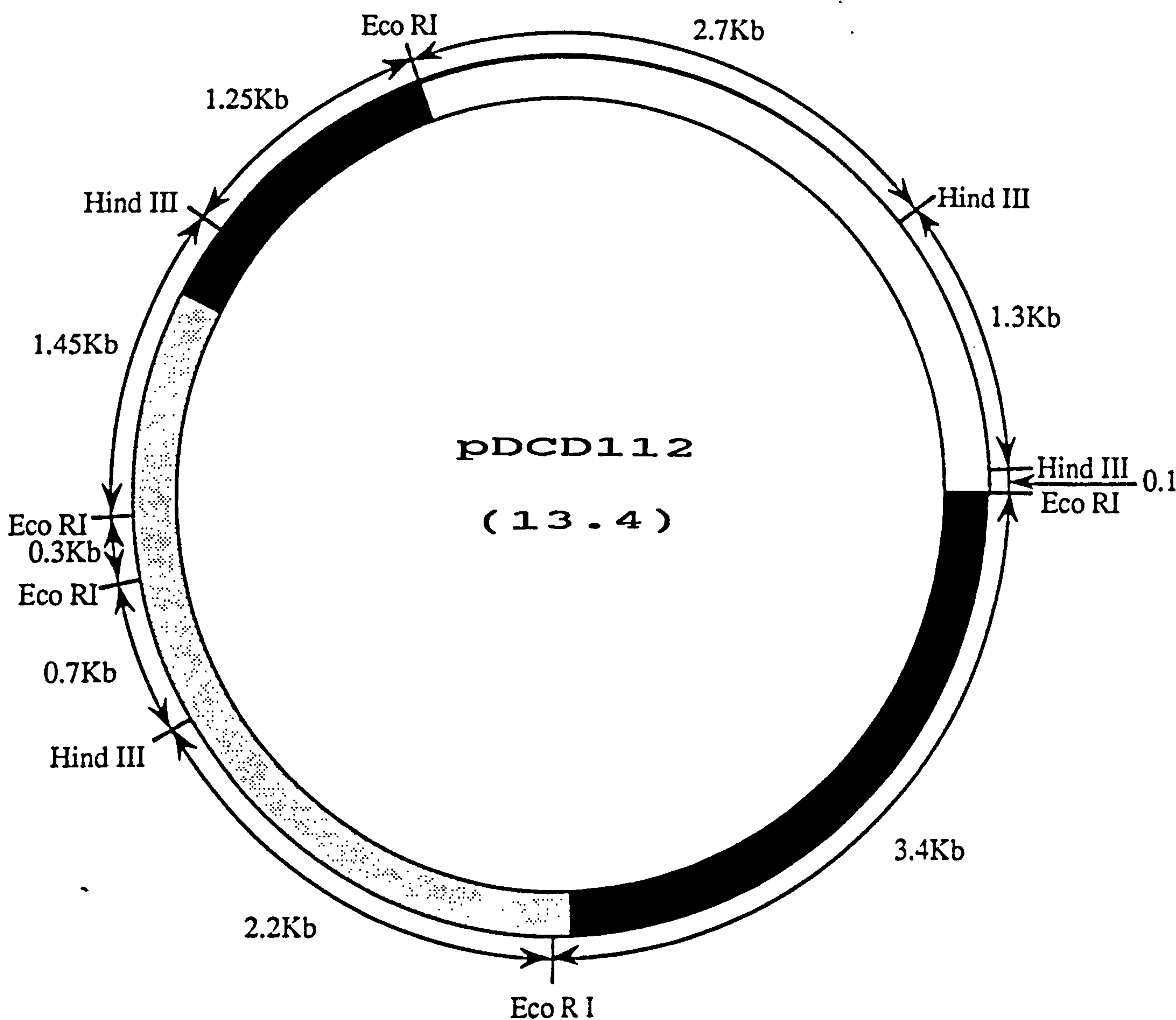
Table 6.2. Cultures of BJ2168R used for RNA Analysis.

Cultures	N° Divisions	OD <sub>600</sub>	Hrs in Culture
Lag Phase	0	0.1	4
Mid Exponential	5	0.2	19
Late Exponential	7-8	0.4	29
Stationary	-	0.8	52

RNA samples were run out on a 1.2% w/v agarose gel containing formaldehyde and blotted onto a Hybond N+ nylon membrane (2.28; 2.29). The fragment of 1.5Kb spanning most of the coding region of the DACH<sub>E</sub> gene obtained by *EcoRI*/*SacI* digest of pKS-DACH<sub>E</sub> (4.2.2) was used as the probe for DACH<sub>E</sub> mRNA on the Northern blot. To check for uniform loading of RNA samples and to assess the amount of DACH<sub>E</sub> mRNA relative to an internal standard of mRNA from a *S. cerevisiae* gene that is constitutively transcribed, the *PDA1* gene (1.3Kb) which encodes the E1 $\alpha$  subunit of the pyruvate dehydrogenase complex from *S. cerevisiae* was used as a control probe (Wenzel et al, 1995). DNA probes were radiolabelled with <sup>32</sup>P  $\alpha$ -dCTP using random priming (Amersham) (2.21.2). Incorporation of radiolabel was measured by TCA precipitation and scintillation counting (2.31) and the specific activities of both probes

Figure 6.1. Plasmid pDCD112 (after Rowe, 1993).





- 4.1Kb Eco RI fragment of yeast 2μ (B form) plasmid DNA
- plasmid pBR328 DNA
- 4.4Kb Bgl II fragment of rDNA from pYir A12

calculated.

Inhibition studies were performed using PMSF and the Ellman assay (2.23). SDS-PAGE and visualisation of proteins by silver staining was used in the analysis of crude protoplast extracts from cultures of BJ2168R and BJ2168W at three stages of the growth curve (Table 6.1) and also in the analysis of protoplast membrane fractions from mid exponential cultures (2.24-2.26). Tunicamycin samples were obtained by addition of tunicamycin at 10 $\mu$ g/ml to mid exponential cultures and allowing cell division to proceed for a further 8 hours. Control cultures of BJ2168R and BJ2168W at mid exponential phase were also allowed to grow for a further 8 hours in the absence of tunicamycin; samples from these cultures could then be used on SDS-PAGE gels both as a control for the tunicamycin experiment and as representative late exponential cultures.

Table 6.1. Cultures of BJ2168R and BJ2168W used for SDS-PAGE Analysis.

Cultures	N° Divisions	OD <sub>600</sub>	Hrs in Culture
Mid Exponential	4	0.1	16
Late Exponential	6	0.4	24
Stationary	-	0.8	48

The membrane fraction of protoplasts was isolated after lysis of protoplasts from mid exponential cultures in 0.5ml 67mM phosphate buffer pH 7.0 by microfuging at 13000rpm for 5 min, discarding the supernatant, and resuspending in 0.5ml fresh 67mM phosphate buffer pH 7.0. Assay was by the Sabine method (2.22.5).

Protoplasts were osmotically stabilized by the presence of 1M sorbitol in the Sabine assay reaction.



Aspects of protoplasting procedure that were examined included increasing the concentration of Zymolyase 20,000 from 1.5mg/ml to 3.0 mg/ml and decreasing the incubation time from 30 min at 37°C to 15 min at room temperature, and testing of the protoplasting solution (minus  $\beta$ -mecaptoethanol) for AChE activity. Protoplasting by the experimental method and the original method was confirmed by 'clearing' in SDW (2.22.3). These experiments were conducted using the Sabine method.

### 6.3. Results

#### 6.3.1. Plasmid Copy Number Estimations

Growth rate analysis of BJ2168R, BJ2168W, and BJ2168 revealed that each strain grew with a division time of 4 hours, indicating that AChE expression does not have a generalised deleterious effect on cellular metabolism. Assessment of plasmid copy number from BJ2168R and BJ2168W further supported this hypothesis since the former was found to have 56+/-11 copies of the expression plasmid per cell and the latter 57+/-13 copies. The autoradiograph of genomic DNA extracted from all three strains, digested with *EcoRI*, blotted and probed using pDCD112, that was subsequently used for densitometry and copy number assessment is given in Figure 6.2. Calculation of copy number in relation to the number of copies of ribosomal RNA genes is given in Appendix 3.1. Expression of the DACH enzyme does not, at least at the levels reported, therefore, adversely affect the stability of the vector.

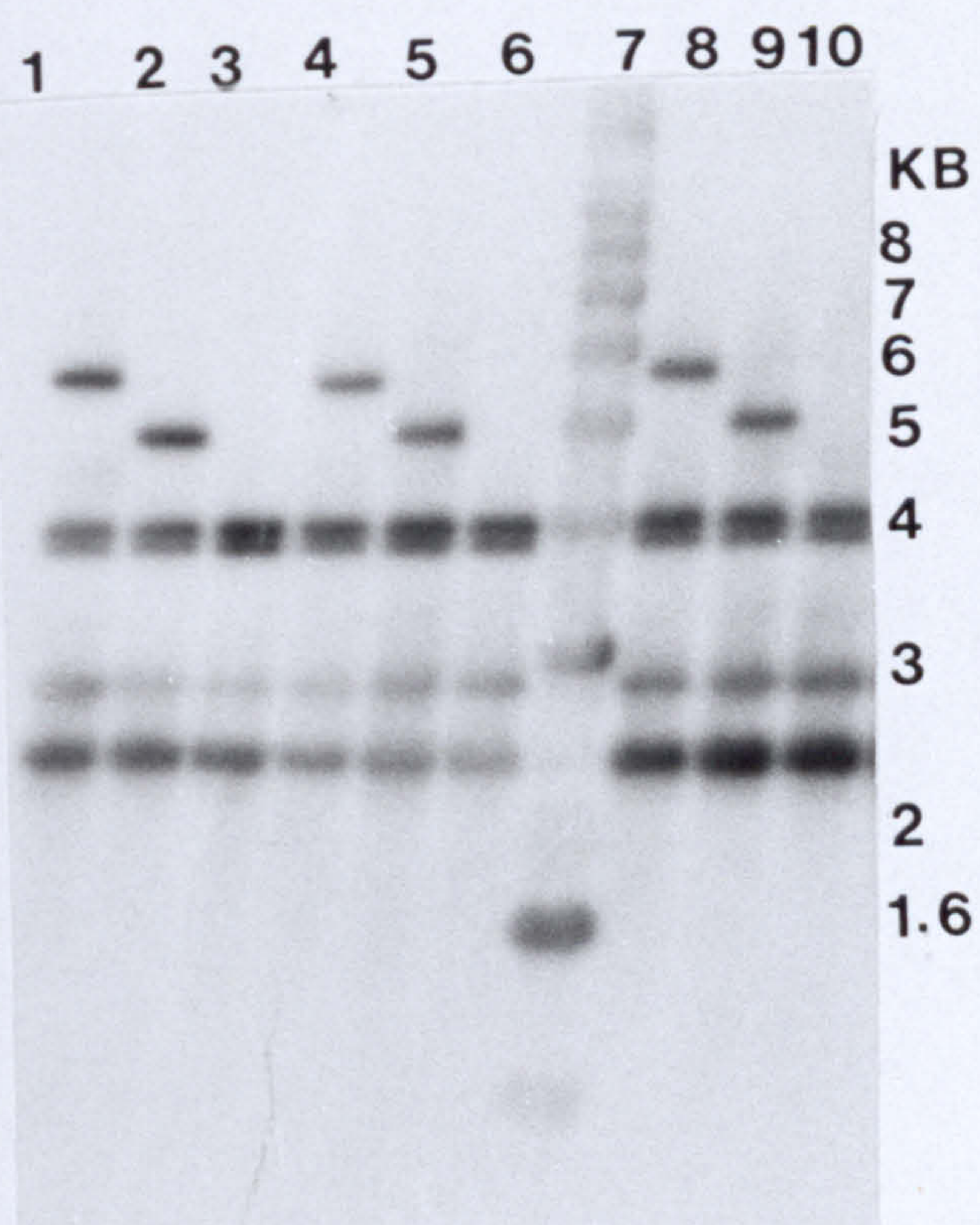
#### 6.3.2. Analysis of mRNA Expression from pG3-DACH in BJ2168R by Northern Blotting

Total RNA was extracted from BJ2168R to examine the level of mRNA expression from construct pG3-DACH. Cultures at

Figure 6.2. Plasmid copy number estimations. A Southern blot of *Eco*RI digested DNA from strains BJ2168R, BJ2168W, and BJ2168 was probed with pDCD112 which detects 2 $\mu$ m DNA (a doublet of approx. 4.1 Kb), rDNA bands (slight homology to 2.8 Kb band and total homology to 2.6 Kb band), and the DAcHE gene by homology with attached pUC18 DNA ( $\approx$  6 Kb in BJ2168R and  $\approx$  5 Kb in BJ2168W). Copy number was calculated using rDNA as a standard of 140 copies per cell (Appendix 3.1). Samples are in triplicate.

Lanes	1, 4, 8	- BJ2168R
	2, 5, 9	- BJ2168W
	3, 6, 10	- BJ2168
	7	- 1Kb Ladder.







four different time points were used (6.2) to assess levels of transcription at different phases of cell growth.

Total RNA was successfully extracted from BJ2168R cultures at all four time points and from BJ2168W cultures at late exponential phase. All RNA samples were undegraded and transfer from the gel to the nylon membrane appeared uniform and efficient (Figure 6.3). 26S, 18S, and 5S ribosomal bands in addition to RNA size markers can be clearly seen. The ruler to the right of lane 12 was used to relate the position of bands on the autoradiograph after probing to the position of ribosomal bands and RNA markers on the original gel.

The expected size of the DACH<sub>E</sub> mRNA was  $\approx$  2700 nt (initiation of transcription in *GPD* promoter approximately 20 nucleotides upstream of 2300 bp DACH<sub>E</sub> cDNA fragment + 380 bp *PGK* 3' untranslated region (Holland *et al*, 1983; Hitzeman *et al*, 1982) and that of the internal standard *PDA1* mRNA known to be  $\approx$ 1300 nt (Wenzel *et al*, 1995); thus the two mRNAs probed in the same hybridisation reaction were expected to be sufficiently well separated on the Northern blot.

A single hybridisation signal was seen at the same position in each lane of the blot after an overnight exposure of the autoradiograph at -70°C (Figure 6.4). The fact that the signal was clearly seen in both control lanes (lanes 10 and 11) suggested strongly that the signal was that of the *PDA1* internal standard. The same signal in each lane was seen at an increased intensity following exposure of a fresh autoradiograph for 5 days at -70°C (Figure 6.5) by which time the outline shadow of the nylon filter could be seen and appropriately marked for measurement (not visible on photograph). The distance from the top of the filter to the middle of each band ( $\approx$  5 cm) was extrapolated back to the original filter (Figure 6.3) placing the position of



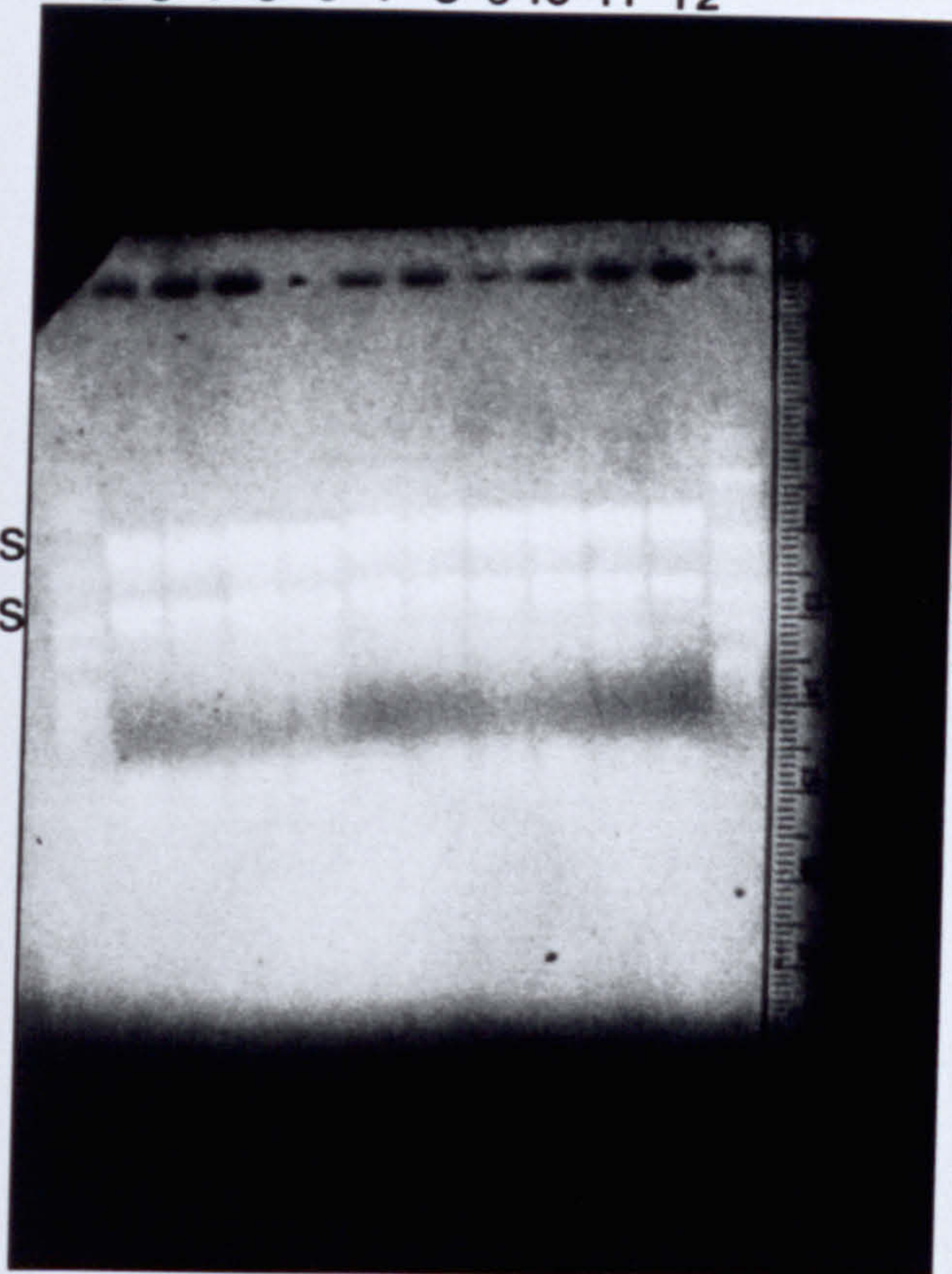
Figure 6.3. Total RNA extracted from BJ2168R and BJ2168W cultures and transferred to a nylon membrane by Northern blotting. Equal amounts (20 $\mu$ g) of total RNA were loaded in each lane. Samples are in duplicate.

Lanes:

1	RNA Molecular Weight Marker I (10 $\mu$ g) (Boehringer)
2-3	BJ2168R (lag phase)
4-5	BJ2168R (mid exponential phase)
6-7	BJ2168R (late exponential phase)
8-9	BJ2168R (stationary phase)
10-11	BJ2168W (late exponential phase)
12	RNA Molecular Weight Marker I (10 $\mu$ g) (Boehringer)

1 2 3 4 5 6 7 8 9 10 11 12

26S  
18S



KB  
5.3  
2.8  
1.9  
1.6  
1.0

26S: 3.8 KB

18S: 2.0 KB



Figure 6.4. Autoradiograph of the Northern blot probed with DACH<sub>E</sub> cDNA and *PDA1* probes (overnight exposure). Lanes correspond to those shown in Figure 6.3.



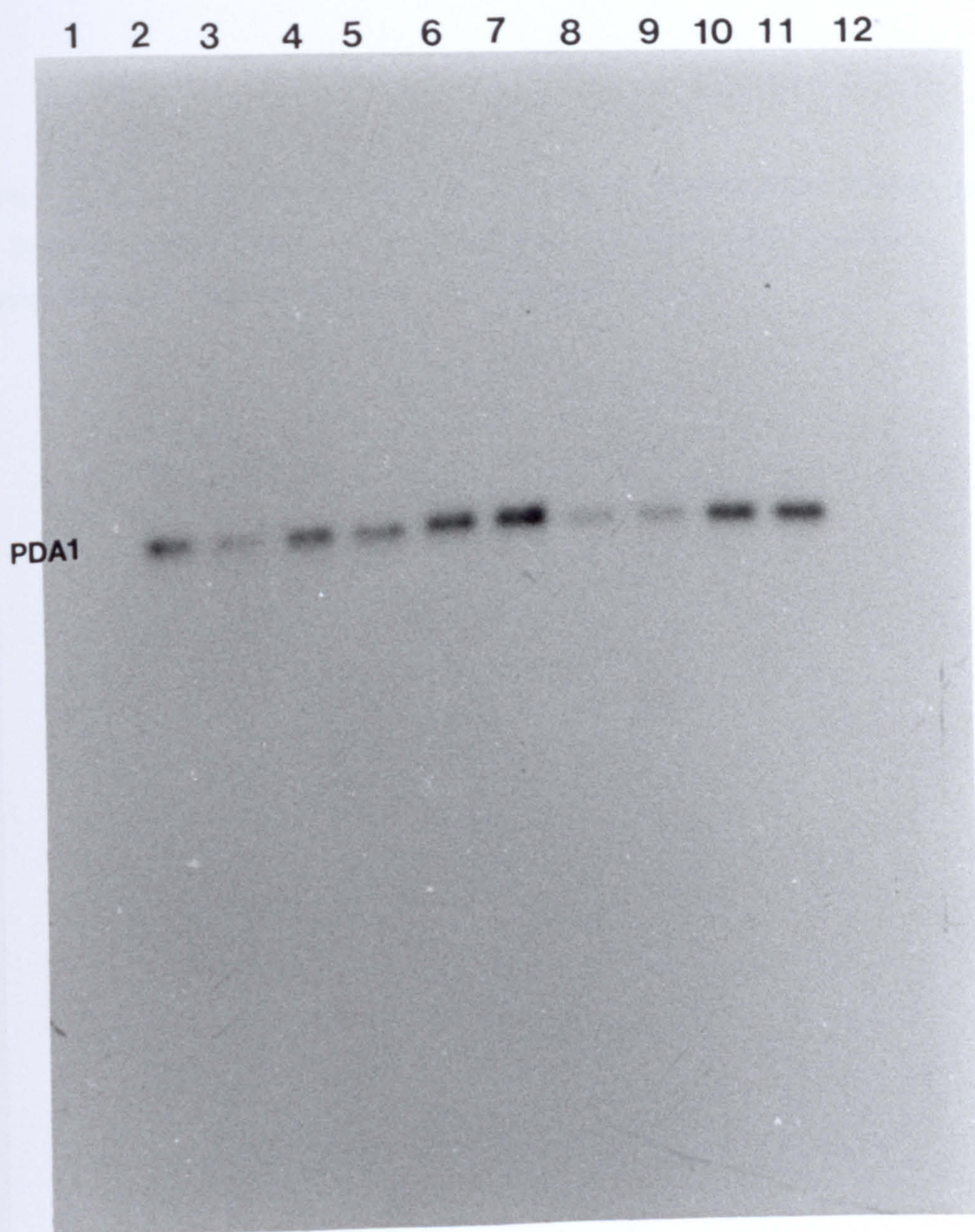
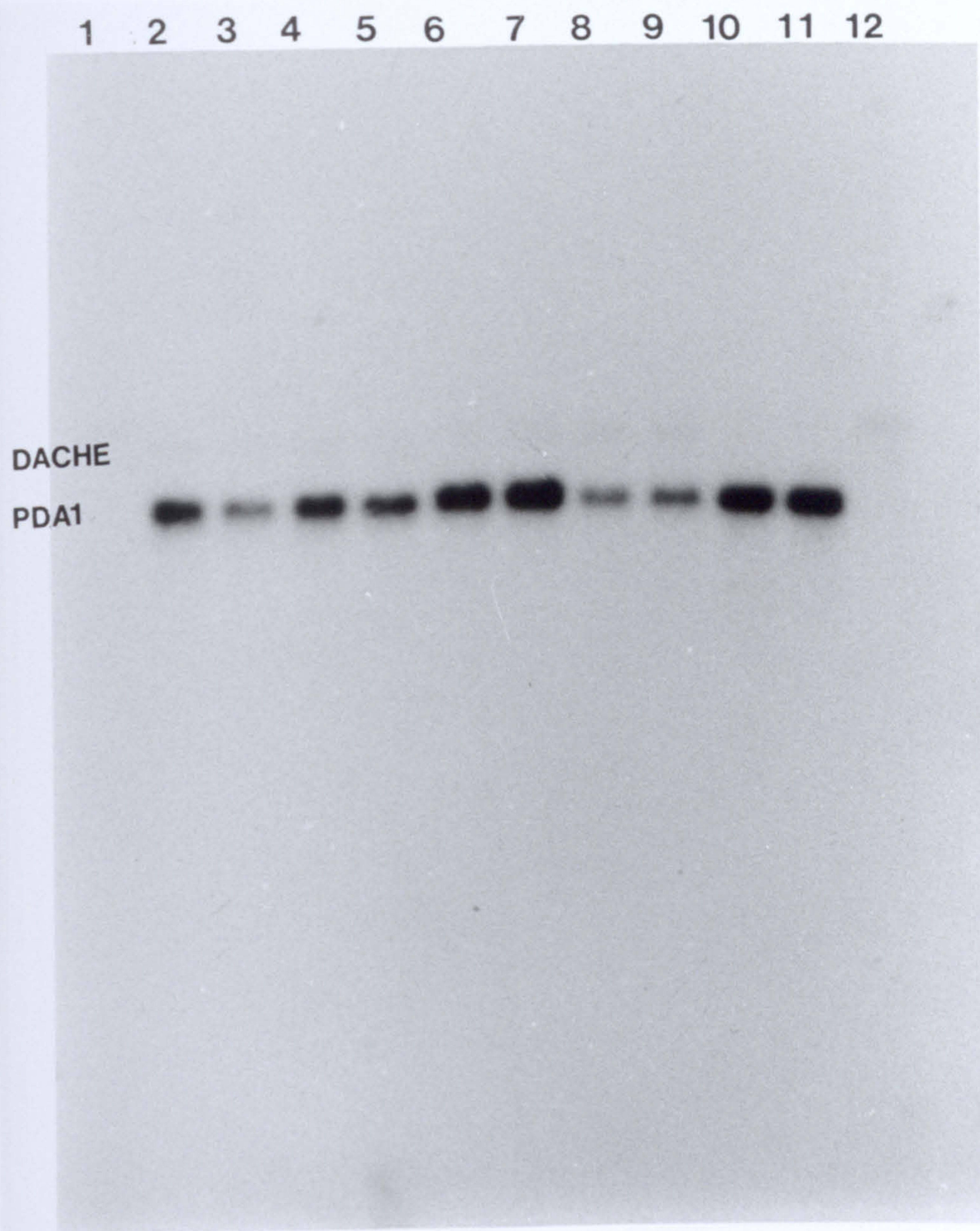




Figure 6.5. Autoradiograph of the Northern blot probed with DACE cDNA and *PDA1* probes (5 day exposure). Lanes correspond to those shown in Figure 6.3.







the hybridisation signals seen at slightly above the 1 Kb marker band. This approximates closely to the known size of the *PDA1* mRNA.

It can be seen that the level of *PDA1* mRNA varies at the different time points spanning the growth curve and that the highest level of expression appears to occur in late exponential phase before declining in stationary phase. A strong signal was still obtained from stationary phase cultures however which is in agreement with results obtained by Wenzel *et al* (1995).

A signal was not obtained for the *DACHE* mRNA after an overnight exposure (Figure 6.4) in contrast to the clear signal seen for the constitutively transcribed *PDA1* gene. A weaker second signal was obtained however after a longer exposure of 5 days (Figure 6.5). This weaker second signal is present in all experimental lanes but noticeably absent from both of the control lanes (lanes 10 and 11). The distance of these weaker bands from the top of the filter shadow on the autoradiograph was found to be  $\approx 4.5$  cm which when extrapolated back to the original filter (Figure 6.3) positioned them slightly above the 1.6 Kb marker band. The expected size of the *DACHE* transcript is  $\approx 2.7$  Kb. Furthermore, following the 5 day exposure some non-specific binding to one of the marker bands (lanes 1 and 12) resulting in a signal of similar intensity to these weaker bands can also be seen on the autoradiograph (Figure 6.5). A longer exposure for 10 days (not shown) failed to highlight any signals other than those clearly seen at 5 days.

With regard to the detection of the *DACHE* transcript the Northern result is interesting. The absence of the weaker signal in both BJ2168W lanes in contrast to its presence in all BJ2168R lanes is strongly suggestive that this is indeed the *DACHE* signal; if so then the transcript detected

is approximately 1000 nt shorter than expected which has implications for the translated protein and its analysis.

A reason for the weakness of the DAcHE signal might have been that the probe had been inadequately labelled. However, calculation of specific activities of both probes based on TCA precipitation and scintillation counting revealed that this was not the case. Specific activities were calculated at  $1.02 \times 10^9$  dpm/ $\mu$ g and  $1.64 \times 10^9$  dpm/ $\mu$ g for the *PDA1* and DAcHE probes respectively (Appendix 3.10).

### 6.3.3. Inhibition with PMSF to Further Investigate Activity and to Estimate Enzyme Concentration

The cholinesterase inhibitor PMSF was used to examine the inhibition profile of the yeast DAcHE and compare it with the profile obtained with a purified AChE from *Torpedo*.

The readout from the microtitre plate reader shows the rate of product formation in the Ellman reaction at three final concentrations of PMSF (1, 2, and 4mM) (Figure 6.6). No inhibition of either the pure or the heterologous AChE was observed at a 1mM final concentration. At a final concentration of 2mM however some inhibition of the *Torpedo* enzyme was seen ( $72.7 \pm 6.50\%$  expressed as a percentage of uninhibited activity levels) but no inhibition of the yeast DAcHE was observed ( $96.3 \pm 7.06\%$ ). This suggests some difference in the affinity of the two enzymes for the inhibitor. However, at a final concentration of 4mM the level of inhibition was found to be similar ( $69.3 \pm 8.46\%$  and  $61.8 \pm 8.19\%$  for the yeast and *Torpedo* enzymes respectively). In contrast a level of activity of  $97.6 \pm 16.52$  was recorded at 4mM PMSF for protoplasts of the control strain BJ2168W (Figure 6.7).

The turnover number for the AChE monomer from electric ray has been calculated at 8000 molecules  $s^{-1}$  (Taylor et al,



Figure 6.6. The effect on the expressed DAcHE from *S. cerevisiae* of the cholinesterase inhibitor PMSF. Non-specific 'mopping up' of the inhibitor by yeast proteins was controlled for in *Torpedo* wells by the addition of BJ2168W protoplasts (2.23).

Group	Well Co-ordinates
BJ2168R Uninhibited	B1 - B3
BJ2168R 1mM PMSF	C1 - C3
BJ2168R 2mM PMSF	D1 - D3
BJ2168R 4 mM PMSF	E1 - E3
BJ2168W Uninhibited	B4 - B6
BJ2168W 1mM PMSF	C4 - C6
BJ2168W 2mM PMSF	D4 - D6
BJ2168W 4mM PMSF	E4 - E6
<i>Torpedo</i> Uninhibited	B7 - B9
<i>Torpedo</i> 1mM PMSF	C7 - C9
<i>Torpedo</i> 2mM PMSF	D7 - D9
<i>Torpedo</i> 4mM PMSF	E7 - E9
Blank	A1 - A9

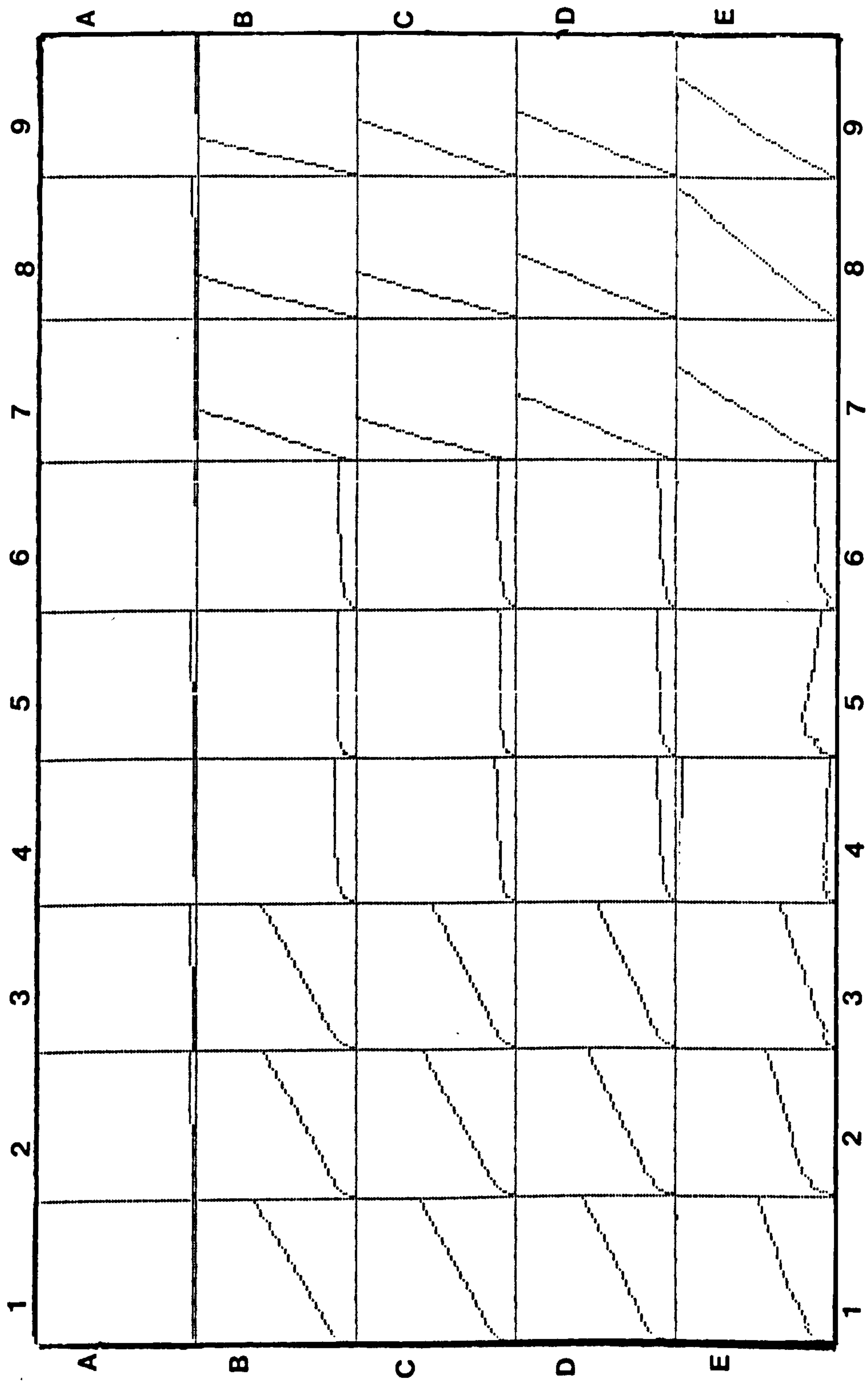
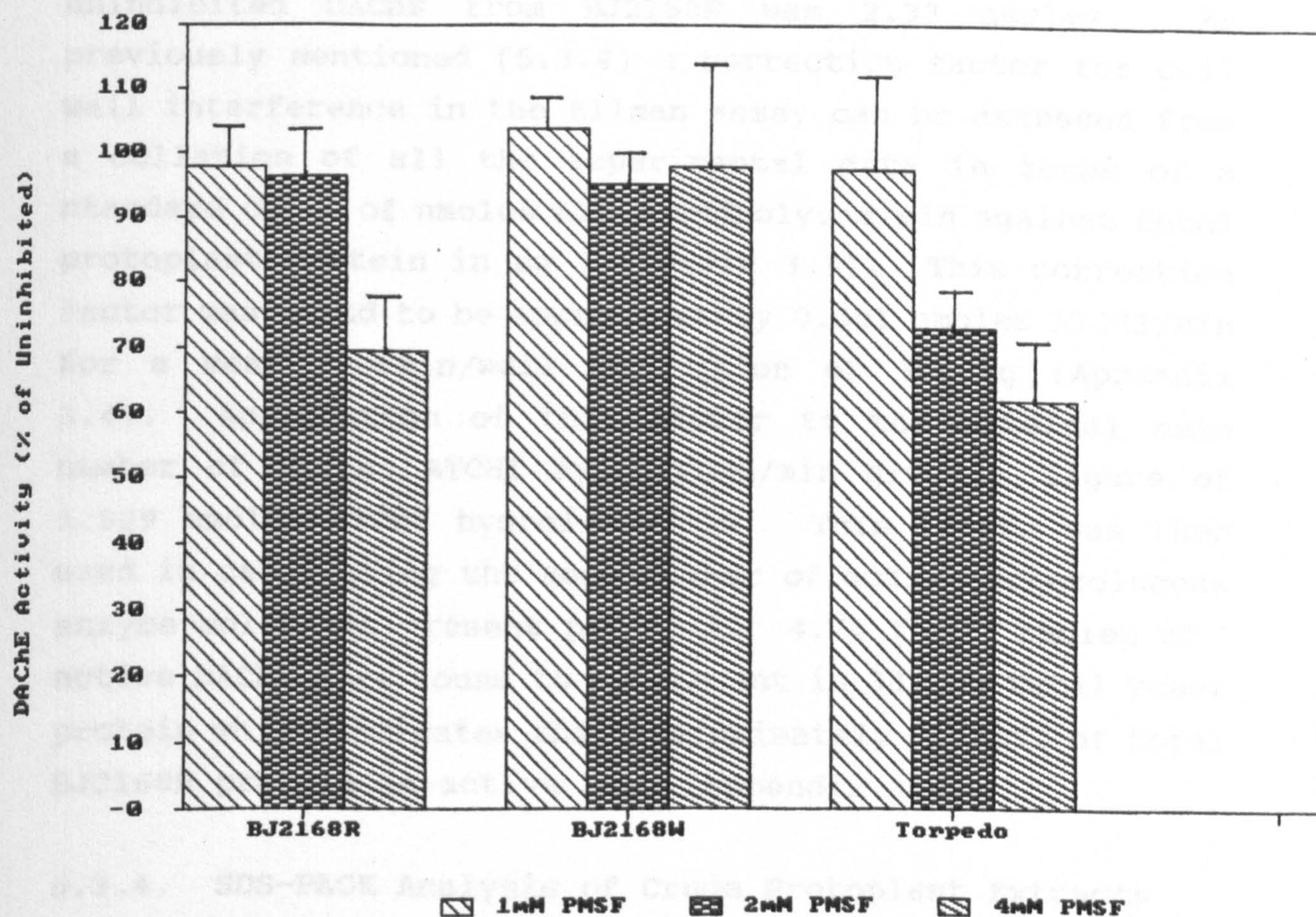




Figure 6.7. The effect of PMSF on the recombinant DACE and *Torpedo* AChE where inhibition is expressed in terms of a percentage of uninhibited activity levels.







1974). Comparison of the levels of inhibition at 4mM PMSF for the *Torpedo* AChE and the DACHe from BJ2168R enabled the turnover number of the heterologous enzyme to be approximated at 6429.3 molecules s<sup>-1</sup> (Appendix 3.4).

The mean number of nmoles ATCHI hydrolysed/min/well by the uninhibited DACHe from BJ2168R was 2.33 nmoles. As previously mentioned (5.3.4) a correction factor for cell wall interference in the Ellman assay can be assessed from a collation of all the experimental data in terms of a standard curve of nmoles ATCHI hydrolysed/min against total protoplast protein in µg (Appendix 3.5). This correction factor was found to be approximately 0.501 nmoles ATCHI/min for a mean protein/well estimation of 38.6µg (Appendix 3.4). Application of this factor to the original mean number of nmoles ATCHI hydrolysed/min gives a figure of 1.829 nmoles ATCHI hydrolysed/min. This figure was then used in calculating the mean number of active heterologous enzyme molecules present per well.  $4.74 \times 10^{-6}$  nmoles of active DACHe were found to be present in 38.6µg total yeast protein which indicates that approximately 0.0009% of total BJ2168R protein is active AChE (Appendix 3.4).

#### 6.3.4. SDS-PAGE Analysis of Crude Protoplast Extracts

The aims of the SDS-PAGE analysis were to visualize the recombinant DACHe monomer subunits in order to assess their molecular weight and compare them with the known weights of AChE from *Drosophila* (55 and 18 KDa (Fournier et al, 1988)) and to obtain an estimation as to the percentage of total recombinant DACHe that was active.

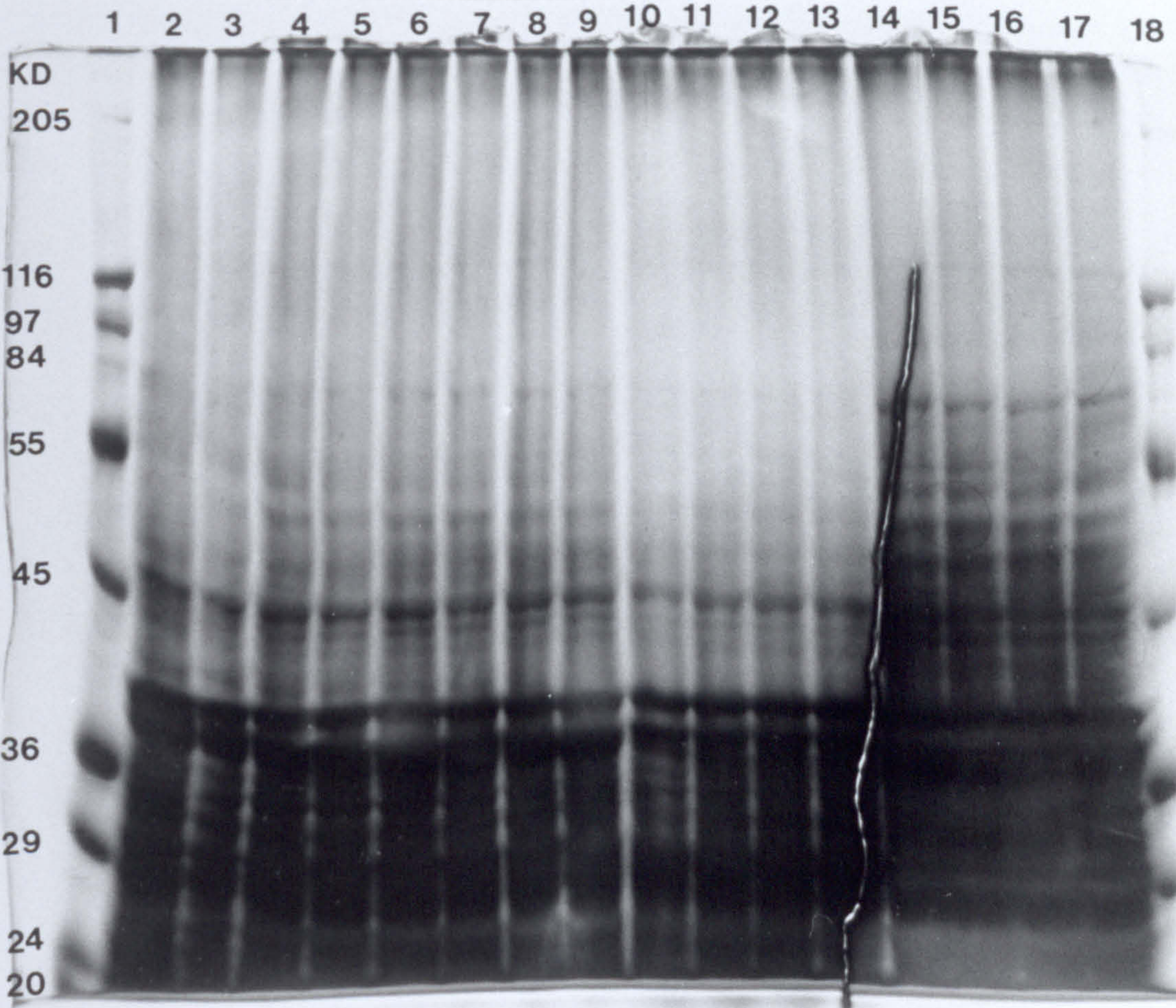
Silver staining of crude protein extracts from BJ2168R and BJ2168W protoplasts run on a 7.5% SDS-PAGE resolving gel failed to highlight the presence of any significant bands present in BJ2168R lanes but absent from control lanes (Figure 6.8). Duplicate samples from both BJ2168R and

Figure 6.8. Crude extracts of BJ2168R and BJ2168W protoplasts analysed by SDS-PAGE (silver stain). Samples are in duplicate. 20µg protein loaded per lane.

Lanes:

1	Protein Molecular Weight Markers (Sigma Wide Range) (4µg)
2 - 3	BJ2168R (stationary)
4 - 5	BJ2168W (stationary)
6 - 7	BJ2168R (mid exponential)
8 - 9	BJ2168W (mid exponential)
10 - 11	BJ2168R (tunicamycin late exponential)
12 - 13	BJ2168W (tunicamycin late exponential)
14 - 15	BJ2168R (tunicamycin control late exponential)
16 - 17	BJ2168W (tunicamycin control late exponential)
18	Protein Molecular Weight Markers (Sigma Wide Range) (4µg).







BJ2168W taken at three different stages of the growth curve were run on the gel (6.2). The optimum concentration of protein (20 $\mu$ g) that allowed the best visualisation of the widest range of band sizes (20 - 200 KDa) and band to background ratio in crude extracts by silver staining was empirically determined. The 7.5% gel gave optimal separation of bands at critical size ranges for the DACH<sub>E</sub> protein under denaturing conditions extrapolated both from the literature (Fournier et al, 1988) and from the Northern analysis which suggested that the recombinant protein may well be truncated (see 6.4).

A significant protein band that was unique to BJ2168R cultures grown in the presence of tunicamycin was not observed (Figure 6.8 lanes 10-13). It can be seen that tunicamycin effected a shift in the mobility of proteins in the SDS-PAGE gel compared to controls, most noticeably in the higher molecular weight range (Figure 6.8 lanes 10-13 compared to lanes 14-17).

The absence of large quantities of enzyme protein suggested that the total amount of expressed DACH<sub>E</sub> was likely to be commensurate with the amount previously calculated for the active enzyme (6.3.3).

#### 6.3.5. Cellular Location of the Recombinant AChE

##### 6.3.5.a. Isolation of the Cell Membrane Fraction of Protoplasts

Analysis of the expression of DACH<sub>E</sub> in *S. cerevisiae* had suggested that activity was associated with the cell membrane/cell wall fraction rather than with microsomal or soluble fractions (5.3.2). To further confirm this the level of activity associated with the cell membrane fraction of protoplasts was assessed and compared with the



level routinely found with whole protoplast extracts. Mean amounts of total protein were found to be 534.4 $\mu$ g for the crude extract of BJ2168R protoplasts prior to microfugation and 93.75 $\mu$ g for the resuspended cell membrane fraction after microfugation (Appendix 3.6.a). Significantly, the mean activity level of the cell membrane extract in terms of nmoles ACHI hydrolysed/min was found to be  $\approx$ 75% of the level recorded for whole protoplast extracts (mean total protein: 492.2 $\mu$ g) (Figure 6.9.a). That  $\approx$ 75% of the expressed enzyme activity was found to be located in the cell membrane fraction of *S. cerevisiae* protoplasts was also reflected in a 4 fold increase in specific activity for the cell membrane fraction over the whole protoplast extract (230.43 $\pm$ 28.19 Units and 60.92 $\pm$ 2.43 Units respectively) (Figure 6.9.b).

#### 6.3.5.b. Analysis of the Cell Membrane Fraction of Protoplasts by SDS-PAGE

The cell membrane fraction of BJ2168R protoplasts, which had been shown to be associated with a higher specific activity of the recombinant DAcHE, was examined by SDS-PAGE to see if the protein could now be visualized.

Four different amounts of membrane fraction protein were loaded on the gel to optimise staining across the wide molecular weight range (20 - 200 KDa). It can be seen that it was not possible to visualize the DAcHE protein despite the very low background level associated with a more purified preparation (Figure 6.10). The low level of background enabled bands to be optimally visualized between 100 and 200 KDa and even above 200 KDa in lanes where lower amounts of protein were loaded (lanes 2-5 and 10-13). Lanes containing higher amounts of protein enabled clear visualisation of bands between 20 and 200 KDa and most importantly in the critical regions (see 6.4).

Figure 6.9.a. Activity levels associated with the cell membrane fraction of BJ2168R protoplasts compared to levels recorded for whole protoplast extracts. Activity levels are expressed as nmoles ACHI hydrolysed/min.

Figure 6.9.b. The mean specific activity associated with the cell membrane fraction of BJ2168R protoplasts compared to that calculated for whole protoplast extracts. Units are defined as nmoles ACHI hydrolysed/min/mg protein.



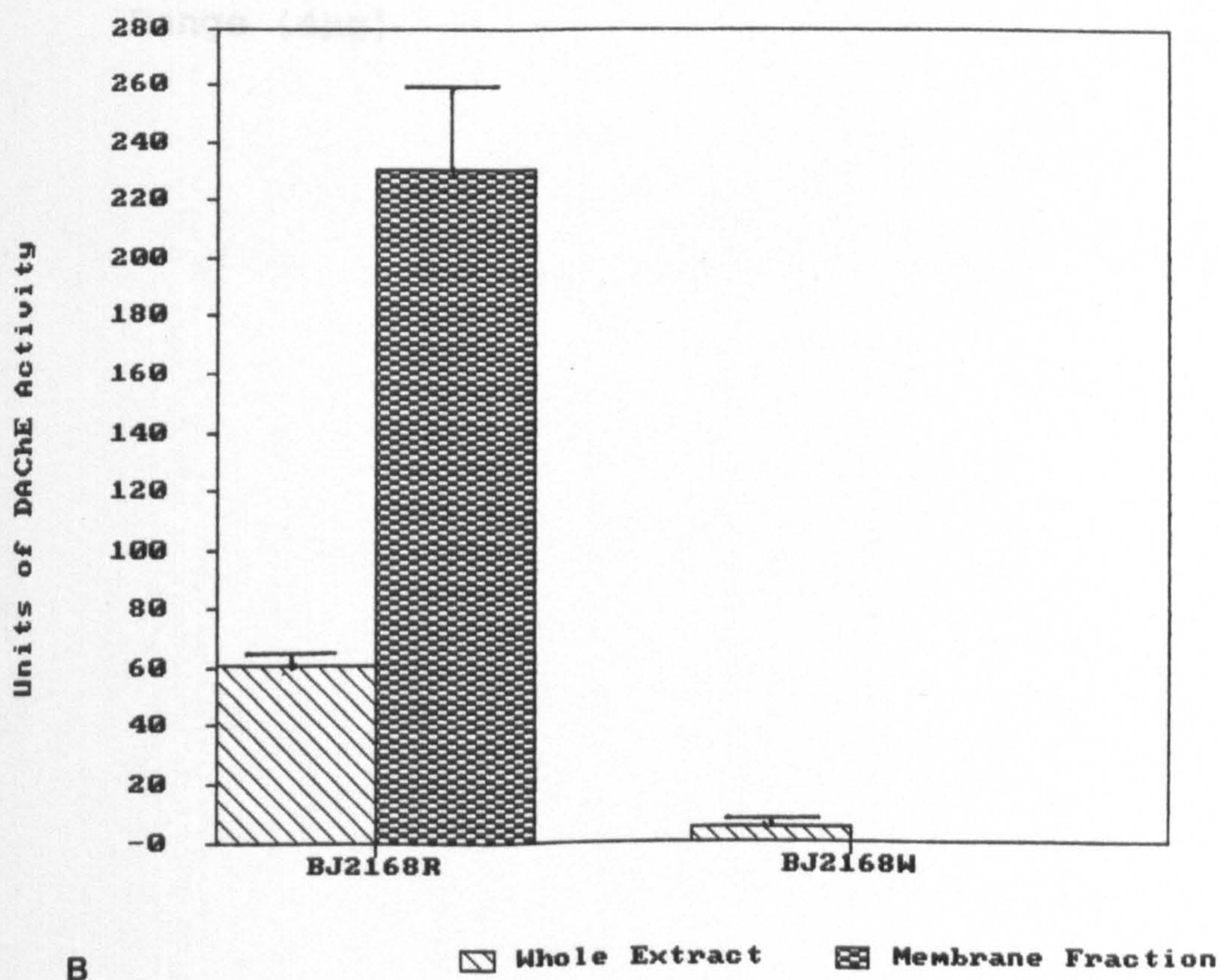
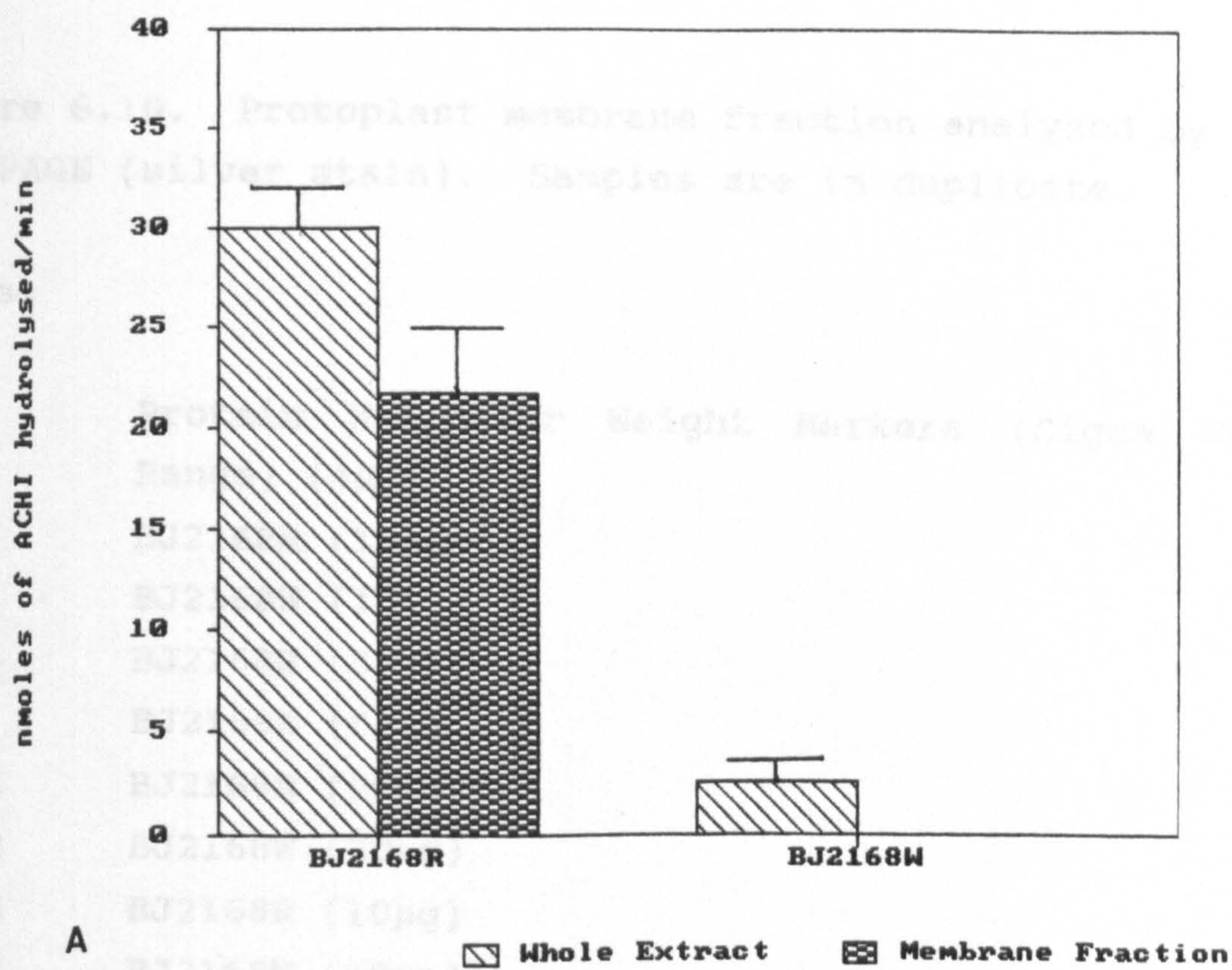


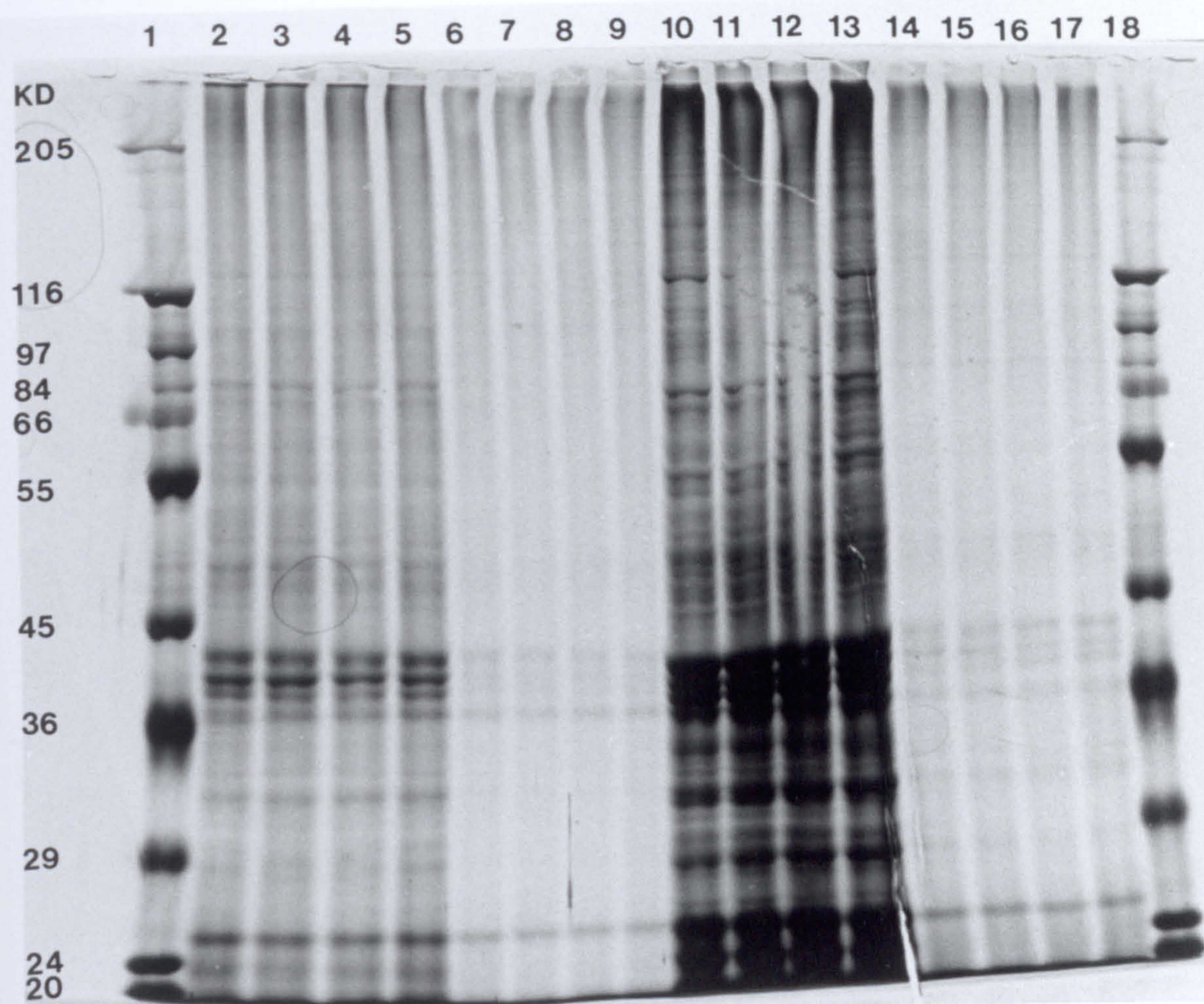


Figure 6.10. Protoplast membrane fraction analyzed by SDS-PAGE (silver stain). Samples are in duplicate.

Lanes:

1	Protein Molecular Weight Markers (Sigma Wide Range) (4μg)
2-3	BJ2168R (15μg)
4-5	BJ2168W (15μg)
6-7	BJ2168R (5μg)
8-9	BJ2168W (5μg)
10-11	BJ2168R (20μg)
12-13	BJ2168W (20μg)
14-15	BJ2168R (10μg)
16-17	BJ2168W (10μg)
18	Protein Molecular Weight Markers (Sigma Wide Range (4μg)







#### 6.3.5.c. Analysis of Osmotically Stabilized Protoplasts

The significant increase in specific activity observed with the cell membrane fraction of BJ2168R protoplasts provided further evidence that the expressed DACH<sub>E</sub> was located in the yeast cell membrane. In its natural form ACh<sub>E</sub> in *D. melanogaster* is linked to the external surface of the neuronal synapse membrane by a glycopospholipid anchor (Fournier *et al*, 1988). Osmotically stabilized protoplasts were used to examine whether the active site of the expressed DACH<sub>E</sub> in the yeast cell membrane was located externally or internally. It was discovered that lysis of BJ2168R protoplasts was required to release activity (Figure 6.11) indicating that the expressed DACH<sub>E</sub> probably resides on the inside rather than the outside of the yeast cell membrane. The slightly higher activity seen with BJ2168R protoplasts in 1M sorbitol over the lysed control is likely to be due to the disruption of a small percentage of stabilized protoplasts during the procedure.

#### 6.3.5.d. Assay of the Protoplasting Supernatant

Certain aspects of the protoplasting procedure were examined in order to investigate the discrepancy observed in activity levels obtained by the two extraction procedures (5.4). The hypothesis that a significant portion of the expressed DACH<sub>E</sub> was being released by the cell into the periplasmic space and that consequently large quantities of enzyme were being lost during the protoplasting procedure was tested by assay of the protoplasting supernatant. Activity was not detected from this fraction, however, (Figure 6.12) indicating that active DACH<sub>E</sub> was not being secreted into the periplasmic space or located in the cell wall.



Figure 6.11. Activity levels of BJ2168R protoplasts stabilized in 1M sorbitol compared to levels typically associated with lysed protoplast extracts. Activity is expressed as nmoles ACHI hydrolysed/min.

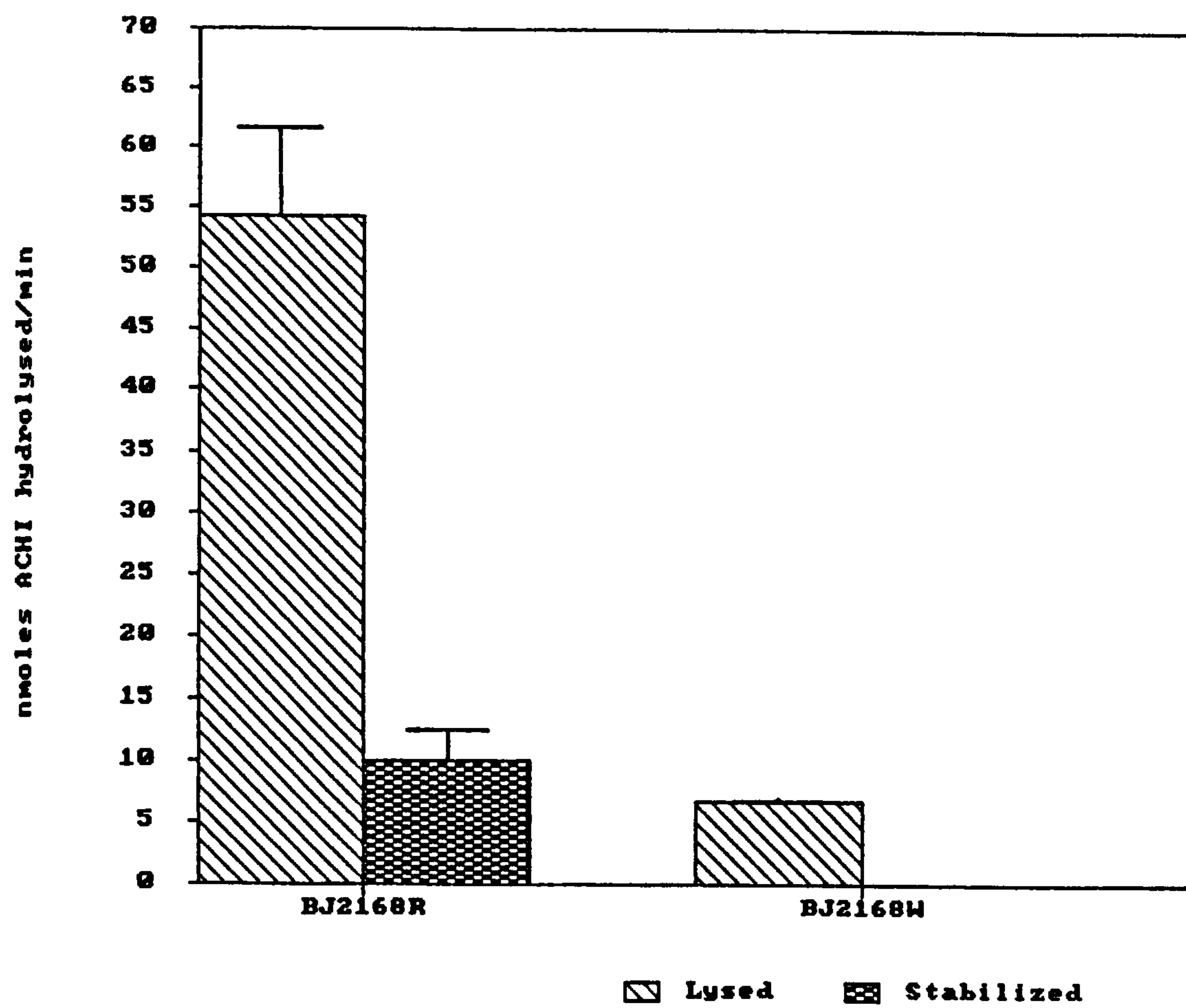
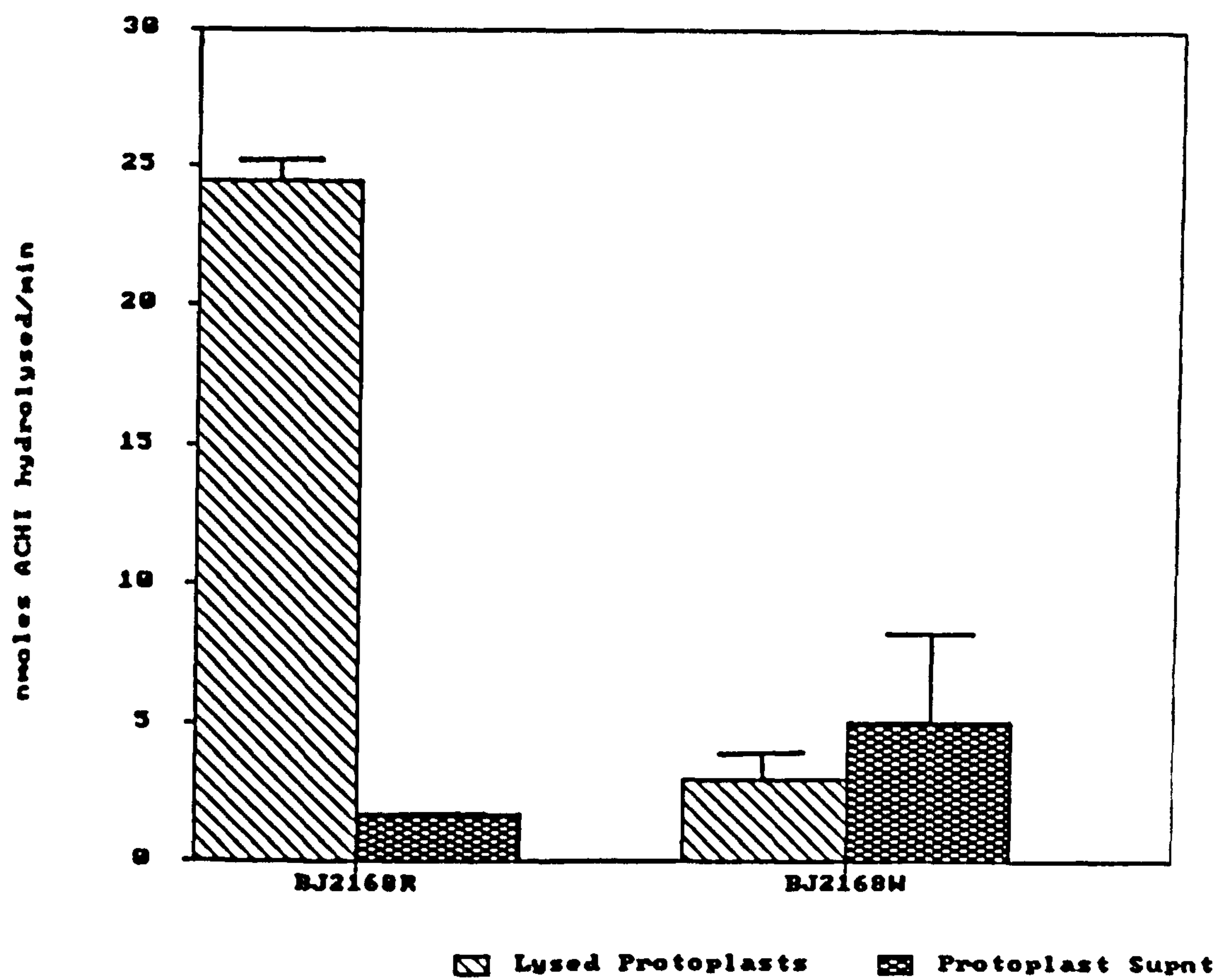




Figure 6.12. Activity levels of the supernatant fraction of protoplast preparations compared to lysed protoplast extracts. Activity is expressed as nmoles ACHI hydrolysed/min.





#### 6.3.6. Alterations in Protoplasting Procedure

The length and temperature of incubation during the protoplasting procedure may have been responsible for a loss of DACE activity. When the concentration of Zymolyase was increased to 3.0 mg/ml to half the incubation time and decrease the temperature of incubation, however, activity levels were largely unaffected (Figure 6.13). A specific activity of  $55.32 \pm 3.30$  Units was obtained which approximates to the  $50.19 \pm 0.67$  Units obtained using the original protoplasting procedure (2.22.3).  $\beta$ -mercaptoethanol was also omitted showing that its presence in the original protoplasting procedure was not responsible for any loss of activity.

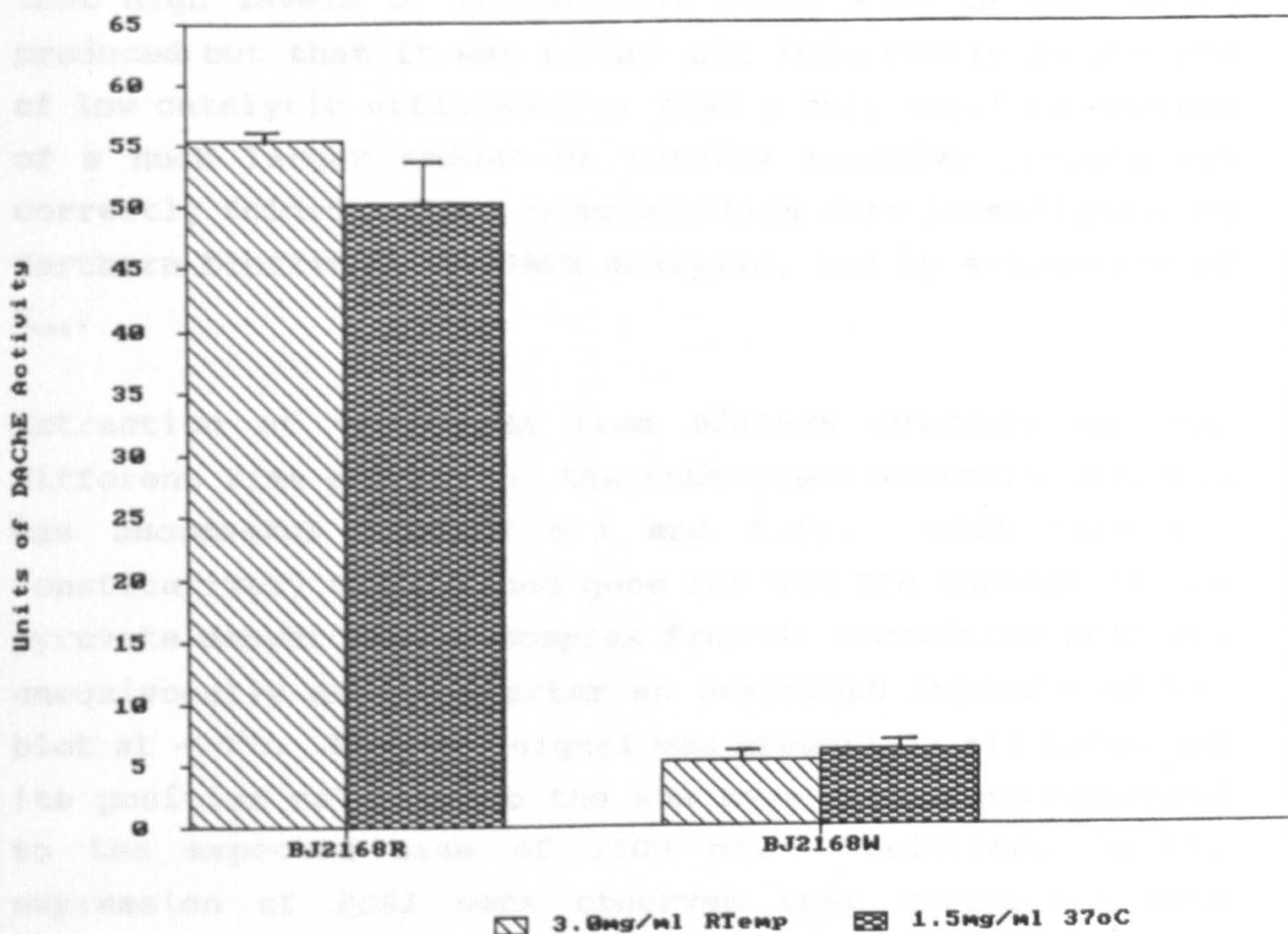
#### 6.4. Discussion

With regard to the long term objectives of the project a yeast expression system for the analysis of insecticide resistant variants of AChE is now available. The levels of DACE expression from BJ2168R protoplasts are well within the limits of detectability by means of the Ellman method and inhibition of the recombinant insect AChE by the cholinesterase inhibitor PMSF was easily observable (Figure 6.6). A higher level of expression would, however, be desirable. Results from plasmid copy number experiments and growth rate analyses indicated that the intracellular expression of AChE does not have an adverse effect on cellular metabolism, leading to a down regulation of the expression vector. Plasmid copy numbers were found to be  $56 \pm 11$  and  $57 \pm 13$  for BJ2168R and BJ2168W respectively (Appendix 3.1) and each strain grew with a division time of 4 hr (data not shown). The attachment of an inducible promoter therefore, is unlikely to increase levels of production.

An unstable mRNA or a low level of transcription may have

**Figure 6.13. The specific activity obtained for protoplast extracts prepared by the standard protocol compared to that obtained for extracts prepared by the altered protocol (see text). Units are defined as nmoles ACHI hydrolysed/min/mg protein.**







been responsible for the low level of expression observed. Conversely a stable DACH<sub>E</sub> mRNA at levels expected from the GPD promoter (equal to or higher than the constitutive control *PDA1*) may have been present but a poor level of translation and/or an unstable protein product may have resulted in low quantities of DACH<sub>E</sub> protein and the low specific activities observed. A further alternative was that high levels of recombinant DACH<sub>E</sub> were in fact being produced but that it was either all incorrectly folded and of low catalytic efficiency or that a only small percentage of a much larger amount of totally inactive protein was correctly folded. These possibilities were investigated by Northern blotting, SDS-PAGE analysis, and by estimation of  $K_{cat}$ .

Extraction of total RNA from BJ2168R cultures at four different time points and the subsequent Northern analysis was successful (Figure 6.3 and 6.4). mRNA from the constitutively transcribed gene for the  $E1\alpha$  subunit of the pyruvate dehydrogenase complex from *S. cerevisiae* *PDA1* was unequivocally detected after an overnight exposure of the blot at -70°C. The *PDA1* signal was present in all lanes and its position relative to the RNA size marker corresponded to the expected size of 1300 nt. Variations in the expression of *PDA1* were observed that correlated with different time points of extraction along the growth curve. It can be seen that signal intensities are approximately equal for BJ2168R and BJ2168W cultures harvested in late exponential phase and that expression of *PDA1* was also highest at this time point (Figure 6.4).

Following a 5 day exposure a second weaker signal was observed that by virtue of its absence in both BJ2168W control lanes contrasted with its obvious presence in all BJ2168R lanes strongly suggested its being the DACH<sub>E</sub> mRNA signal (Figure 6.5). Furthermore, the approximately equal intensities of the *PDA1* bands in the control lanes and



experimental lanes at late exponential phase provides a good control for uniform loading of RNA. Some binding at approximately the same intensity was observed to one of the RNA marker bands in both flanking lanes. Since no other non-specific binding was observed it is likely that this is due to a degree of shared sequence (either a short stretch of totally homologous sequence or a longer stretch of partial homology) between the marker transcript and one of the radiolabelled probes. The Boehringer Mannheim molecular weight transcripts are produced by *in vitro* transcription of linearized plasmids with either SP6 or T7 RNA polymerases in separate reactions. Unfortunately the sequences of the central portions of these transcribed plasmids are not available for comparison (the sequences are 'protected' by Boehringer Mannheim).

The point of a control is that it functions as such. On this assumption the second weaker signal on the Northern blot is the DAcHE transcript. The result has two important implications. Firstly, the DAcHE signal is markedly weak in comparison to the *PDA1* signal (approximately an order of magnitude), with mRNA levels far below that of the constitutively transcribed gene and secondly the DAcHE transcript is sized at  $\approx 1.7$  Kb which is around 1000 nt shorter than expected. It is also of interest that the transcript levels appear fairly constant at all four time points indicating a similar level of DAcHE expression throughout the growth curve.

The DAcHE cDNA in pG3 should possess an efficient transcriptional terminator conferred by the 3' untranslated region from PGK (380 bp) (Hitzeman *et al*, 1982) and the absence on the Northern blot of additional transcripts of heterogeneous length suggest that the terminator is operating efficiently. However, multiple parameters influence the stability of an mRNA in yeast, the precise mechanisms of which are still poorly understood. They

include not only the efficient termination of transcription so that production of long and unstable transcripts is prevented, but also the addition of a poly(A) tail of suitable length (this tail in yeast is of highly heterogeneous length between 25 - 80 adenines (Piper and Aamand, 1989) in addition to the 5'-cap, and various structural determinants which lie within the mRNA. Secondary structure in particular is known to have an effect on both mRNA stability and the efficiency of translation (Loison, 1994; Ross, 1995).

Secondary structure is known to play a role in mRNA turnover in terms of recognition by ribonucleases (Ross, 1995). It is conceivable that an inappropriate hairpin or bulge structure could form in the 3' untranslated region (UTR) upstream of the terminator region conferred by the PGK sequence (there is approximately 230 nt of 3' UTR in the final 2.3 Kb fragment cloned into the Sac I site of pG3). This hairpin could be preferentially cleaved by an endonuclease leading to rapid degradation from the 3' end by an exonuclease. It is known, for example, that mutations in the 3' UTR of genes can lead to considerable variations in transcript stability (Ross, 1995; Tang et al, 1997). Although a low level of transcription from the GPD promoter cannot be ruled out, it seems likely, particularly in the light of a putative truncated transcript, that instability of the DACH<sub>E</sub> mRNA relative to that of the constitutive control is, at least in part, responsible for the relatively low expression observed from pG3-DACH<sub>E</sub> in BJ2168R.

The expected size of the DACH<sub>E</sub> transcript was 2.7 Kb in contrast to the 1.7 Kb transcript detected on the Northern blot. Clearly, the fact of its detection argues for a degree of stability above that of the full length transcript. Once again secondary structure could be conceived as responsible in terms of halting or slowing



down the degradation of the transcript at a particular point. Alternatively, it could be argued that secondary structure at this point in the sequence could cause the yeast RNA polymerase to pause and perhaps dissociate from the template without completing transcription.

The fact that the recombinant DACE exhibits activity also argues for degradation from the 3' rather than the 5' of the transcript since it provides good evidence for the presence of the active site (particularly the catalytic triad). Assuming degradation from the 3' end and taking into account the 380 bp stretch of PGK 3' UTR results in  $\approx$  620 nt of sequence eliminated from the 3' end of the DACE transcript to give an approximate 1.7 kb transcript length. Translation of a transcript of this length 5' to 3' would terminate approximately at position 2700 (from Figure 4.1 and 4.2) and would include the consensus sequence around the active site serine-276 (FGESAG) (position 1900-1930 Figure 4.1) and histidine-518 (position 2638 Figure 4.1) of the catalytic triad. All other residues known to be important for the catalytic activity of AChE including the third member of the catalytic triad, glutamate-405, and residues of the anionic subsite and active site gorge such as tyrosine-109, phenylalanine-368, and tryptophan-121 would also be encoded by the 1.7 Kb transcript. Such a putative AChE polypeptide would be truncated by approximately 130 amino acids upstream of the native terminator. The last 30 amino acids are thought in *Drosophila* to be involved in signalling replacement of the C-terminus with the glycolipid anchor in the ER (Fournier *et al*, 1992) and are therefore not at all crucial to polypeptide folding or catalytic activity. Similarly it can be envisaged that a further proportion of the C-terminal end attached to the glycolipid anchor may not be fundamental to the enzyme in terms of folding that is directly related to catalytic activity.

A spontaneous *in vivo* DNA deletion of  $\approx$  1Kb within the DACH<sub>E</sub> gene may also have been responsible for the truncated transcript observed. However, results from the plasmid copy number analysis argue strongly against this since the size of the plasmid following *Eco*RI digestion is as expected (6.01 Kb) (Figure 6.2). This plasmid fragment from BJ2168R detected by homology of pDCD112 with pUC18 sequences contains a large portion of the cloned DACH<sub>E</sub> gene (1.48 Kb from the 3' end of the gene to the *Eco*RI site towards the 5' end) (from Figure 4.2) in addition to the PGK terminator (Figure 4.12). In Figure 6.2 the plasmid fragment appears slightly below the 6Kb marker but it should be noted that the exact size of the 6 Kb marker band in the 1 Kb ladder is 6.108 (Gibco-BRL). A deletion encompassing the 5' end of the gene, which would also remove the internal *Eco*RI site, is unlikely to be compatible with the expression of an active DACH<sub>E</sub> product since a deletion or degradation of the transcript by 1 Kb from the 5' end effectively eliminates serine-276.

In light of the relatively low DACH<sub>E</sub> transcript levels highlighted by the Northern analysis it was unlikely that the low activity observed from the recombinant DACH<sub>E</sub> was due to large quantities of inactive enzyme since a low level message is often correlated with a low level of translated product. A relatively large amount of inactive product still remained a possibility, however, if the translation efficiency was extremely high, in short that the low level of DACH<sub>E</sub> transcript observed was not due to instability or poor transcriptional efficiency but to a rapid turnover to translated product.

SDS-PAGE analysis of crude protoplast extracts did not reveal the presence of large quantities of DACH<sub>E</sub> protein. Silver staining of SDS-PAGE gels failed to highlight any protein bands in BJ2168R lanes that were absent from BJ2168W lanes (Figure 6.8). This is in agreement with



recent findings that large amounts of inactive protein do not appear to be associated with the expression of rat and *Bungarus* AChE in *P. pastoris* (Morel and Massoulie, 1997) and contrasts acutely with the intense protein band reported by Fischer et al (1993) that is seen in total cell lysates of *E. coli* expressing human AChE analysed by SDS-PAGE and stained with Coomassie blue (sensitivity at least an order of magnitude less than silver) and which is conspicuously absent from control lanes. These points are discussed in detail in 8.1. Cultures of BJ2168R and BJ2168W at three different time points along the growth curve were examined and the optimum amount of protein that gave the best visualisation of the broadest range of band sizes (20 - 200 KDa) for crude extracts stained with silver is shown in Figure 6.8.

It is unlikely, therefore, that quantities of active DACH<sub>E</sub> from *S. cerevisiae* could be significantly raised by solubilization and refolding of the enzyme following purification.

The recombinant enzyme may not be processed in yeast in a similar manner to the post-translational modifications undergone in its native host. For example the monomer (dimers and above are reduced under SDS-PAGE denaturing conditions) may not be cleaved into two separate polypeptides of 18 and 55 KDa (that are then non-covalently associated) but may migrate as a single polypeptide of  $\approx 73$  KDa. This assumption is based upon the translation of a full length transcript, which despite the evidence of the Northern blot still remains a possibility, since very low levels of a full length DACH<sub>E</sub> mRNA may have remained undetected (see 8.2). However, it was also vital to examine the possibility that a truncated polypeptide product was present. Translation of a 1.7 Kb transcript (actually  $\approx 1.6$  Kb of sequence downstream of the start of translation) would result in a polypeptide of  $\approx 60$  KDa.

This truncated polypeptide would also include the hydrophilic segment from position 140 to position 180 which is the site of cleavage in *Drosophila* into the two non-covalently associated subunits. Therefore, further cleavage of the truncated product into polypeptides of  $\approx 18$  and  $\approx 42$  KDa was also a possibility.

All critical size ranges of subunit (with the possible exception of an 18 KDa polypeptide, but see below) are contained and sufficiently well separated (as evidenced by the marker bands) on the SDS-PAGE gel to highlight the recombinant protein if it were present in significant quantities.

A further factor that may affect protein migration on a SDS-PAGE gel is glycosylation. In *Drosophila* the AChE protein is modified by glycosylation of 4 asparagine residues (Mutero and Fournier, 1992). In *S. cerevisiae* the pattern of glycosylation may be different, for example the protein may be hyperglycosylated (see 8.2) which could retard its migration in the gel. In short a 60 KDa band, for example, may run at a significantly higher molecular weight. However, analysis of BJ2168R cultures grown in the presence of tunicamycin (Figure 6.8 lanes 10-13) suggests that although this may well be the case the protein is still not visible as a significant band.

Activity levels associated with the cell membrane fraction of BJ2168R protoplasts were found to be  $\approx 75\%$  of those associated with whole protoplast extracts which provides strong evidence for the cellular location of the expressed DACH<sub>E</sub> (Figure 6.9.a). Furthermore it is likely that of the  $\approx 25\%$  drop in activity seen with the cell membrane fraction some was due to the loss of enzyme molecules to the supernatant fraction during protoplast lysis. This fraction was subsequently discarded to allow resuspension of the isolated cell membrane fraction (6.2). The probable



location of the expressed DACH<sub>E</sub> to the cell membrane was confirmed by the observed four fold increase in specific activity associated with this fraction (Figure 6.9.b.).

The percentage of total *S. cerevisiae* protoplast protein that was found to be active recombinant DACH<sub>E</sub> was 0.0009% (Appendix 3.4). The SDS-PAGE analysis of membrane fractions suggests that this is probably commensurate with the amount of actual DACH<sub>E</sub> protein translated. The evidence from the analysis of crude extracts had indicated that the recombinant protein was not present in large inactive amounts in *S. cerevisiae* and the increased specific activity associated with the cell membrane fraction (Figure 6.9.b) suggested that analysis of this fraction may highlight the recombinant AChE band.

This purification step had two major advantages over the analysis of crude extracts. Firstly, the DACH<sub>E</sub> protein should theoretically be present as a higher proportion ( $\approx 4X$ ) of the total protein population (which is itself significantly reduced allowing clearer band separation) and secondly a purer preparation significantly reduces background in the silver stain. The enhanced signal to noise ratio enables different quantities of protein to be loaded on the gel to optimize the visualisation of bands across a wide range of molecular weights. The loading of smaller protein amounts can help to clarify bands containing very small quantities of protein that may merge and become less sharp when higher amounts of protein are loaded. When crude extracts are stained with silver the loading of smaller protein amounts often results in the loss rather than the sharpening of bands due to the higher background levels. The loading of different amounts of membrane protein enabled clear visualisation of protein bands between 20 and 200 KDa and even above 200 KDa (Figure 6.10). All critical size ranges approximating to 42, 55, 60 and 73 KDa are clearly examined by the analysis and even

very large migrational changes allowed for since clearly defined bands can be seen well above 200 KDa in lanes where lower protein amounts were loaded (Figure 6.10 lanes 6-9; 14-17). It is possible that a polypeptide migrating at  $\approx$  18 KDa would not have been sufficiently well separated and would probably run with the dye front at the acrylamide concentration used (Figure 6.10). However, the acrylamide percentage was chosen to allow sufficient separation of a very wide range of molecular weights (between 20 and 200 KDa) and to allow good separation of bands at sizes critical for resolution of the larger polypeptide (42 or 55 KDa) or an uncleaved polypeptide (60 or 75 KDa) both of which would contain the active site, evidence for the presence of which, is provided by the observation of activity. Furthermore, the smaller cleaved polypeptide may well have been resolved at the dye front if present in sufficient quantity and also resolved if running higher due either to an altered glycosylation pattern or if the signal peptide of 38 amino acids remains uncleaved from the 18 KDa polypeptide resulting in a weight approximating to 22 KDa and contained within the marker range. Despite this the DAcHE protein was not highlighted.

The sensitivity of the silver stain is usually estimated at 1 - 10 ng protein per band. If the estimate of total recombinant protein is  $\approx$  0.0009% then in the cell membrane fraction this should approximate to 0.0036% of the protein population loaded on the gel. For 20  $\mu$ g of total cell membrane fraction protein this represents  $\approx$  0.72ng for the DAcHE protein band. It can be seen that the quantification of DAcHE based on the PMSF study places detection of the recombinant protein band at the limits of resolution by silver staining.

The  $K_{cat}$  value calculated for the expressed DAcHE (6429.3 molecules  $s^{-1}$ ) approached the value reported by Taylor et al (1974) for *Torpedo* (8000 molecules  $s^{-1}$ ) and was similar to



the  $K_{cat}$  of 6666.7  $s^{-1}$  reported by Kronman et al (1994) for human AChE, indicating an AChE of high catalytic efficiency. The evidence from the PMSF inhibition study and the SDS-PAGE investigations effectively rule out the possibility of a large amount of enzyme of low catalytic efficiency. The presence of large quantities of completely inactive enzyme in addition to small quantities of enzyme approaching native levels of efficiency can also be disregarded in the light of the SDS-PAGE results. The data suggest that the small amounts of recombinant DACHe produced in *S. cerevisiae* consist of an enzyme in a conformation capable of a substrate turnover similar to that of *Torpedo* AChE. If the DACHe produced is indeed truncated then the deleted amino acid sequence towards the C-terminal end does not appear to greatly affect conformation, at least in regard to catalytic activity.

The low level of final protein product appears at least in part to be due either to an unstable DACHe mRNA or poor transcriptional efficiency. In view of the high plasmid copy number and an observed truncated transcript the former possibility seems more likely. Absence of sufficient stability of the DACHe truncated transcript to allow for high levels of translation would appear to be a major problem in the accumulation of significant quantities of final AChE product. A problem with the actual efficiency of translation itself does remain a possibility, but the poor transcript yield suggests otherwise, and furthermore the upstream leader sequence of the DACHe cDNA containing AUG codons (which are known to inhibit translation in yeast) is deleted in the construct pG3-DACHe and it seems reasonable to assume that a 5' methylated cap structure important for the initiation of translation would be attached to the DACHe transcript since natural yeast mRNAs are capped at the 5' end. However, stability of the final protein product is also a crucial parameter in determining the overall level of production. Despite the observation

of activity which argues for a more or less correct conformation in terms of the active site it is still possible that a truncated protein may be considerably more susceptible to degradation (see 8.2). This may also be a contributory factor in the low production level seen.

The active site of the DAcHE in the cell membrane of *S. cerevisiae* is probably located internal rather than external to the membrane because lysis of protoplasts was found to be required for the release of activity. BJ2168R protoplasts that had been osmotically stabilized were found to be incapable of substrate hydrolysis (Figure 6.11). Had the active site been located on the outside of the membrane or the DAcHE linked to the external surface of the membrane as naturally in *D. melanogaster* then at least some substrate hydrolysis by stabilized protoplasts would have been expected.

The location of a putative truncated DAcHE in the yeast cell membrane is interesting since in the native host it is the hydrophobic tail of 29 amino acids at the C-terminus which has been postulated to transiently retain the protein at the luminal surface of the ER prior to attachment of the glycopospholipid anchor (Low, 1987; Fournier et al, 1992) and when not cleaved from the mature protein, due to incorrect processing, to act as an ER retention signal (Fournier et al, 1992). However, absence of this hydrophobic region does not preclude an association with the membrane in *S. cerevisiae* since there is an additional hydrophobic region within the protein (Figure 4.1) and it is also possible that the hydrophobic signal peptide at the amino terminus thought to be necessary for transport across the ER membrane in *Drosophila* may not be cleaved from the mature protein in yeast (see 8.2).

Reasons for the discrepancy observed between activity levels associated with lysed protoplasts and enzyme



extracted using glass beads (5.4) remain unclear. A major factor may have been loss of enzyme from the periplasmic space or cell wall during the protoplasting procedure, but testing of the protoplasting solution showed that activity was not being lost during Zymolyase digestion (Figure 6.12). The hypothesis that many DAcHE molecules become trapped in membrane micelles on rupturing of protoplasts effectively internalising the enzyme seems unlikely since the inclusion of Triton X-100 at a final concentration of 0.1% v/v in the lysis buffer and microtitre plate wells has no significant effect on activity (data not shown). The inclusion of sodium deoxycholate also has no effect (Leische, 1996). The presence of the expressed enzyme in the periplasmic space is also inconsistent with its probable location on the inside rather than the outside of the cell membrane.

Neither the temperature nor the length of incubation during the protoplasting procedure appear to be responsible for any significant loss of activity. Reduction of the temperature to ambient room temperature and halving of the period of incubation with Zymolyase resulted in similar levels of activity to those seen with the original procedure (Figure 6.13).  $\beta$ -mercaptoethanol used in the original method should be effectively eliminated by the discarding of the protoplasting solution and subsequent washing of the protoplasts (2.22.3) and its complete omission from the experimental procedure provides further evidence that its use does not affect activity levels.

## CHAPTER SEVEN GENERATION OF A HOMOLOGOUS AChE PROBE FROM *CULEX MOLESTUS*

### 7.1. Introduction

Successful expression of DAcHE from construct pG3-DAcHE in *S. cerevisiae* demonstrated the feasibility of using a yeast expression system for the study of insecticide resistant variants of AChE from mosquitoes.

A fully susceptible mosquito AChE cDNA was desirable as a starting point for the generation of specific mutations which might give rise to degrees of resistance. A strain of fully susceptible autogenous *Cx. molestus* (Elephant) (a member of the *Cx. pipiens* species complex) originally isolated from the London Underground and reared in the laboratory was a suitable source.

The isolation of an AChE cDNA from a potential *Cx. molestus* cDNA library required a suitable probe. A genomic clone containing the AChE gene from a resistant strain of *Cx. pipiens* from Southern France was available and its sequence had been partially determined (Rooker, 1994). Sequence was available for exons 2 and 3 of the gene (as described by Hall and Malcolm, 1991 for *An. stephensi*) and for introns either side and between. Excision of a fragment containing these exons was possible but would have given rise to a fragment containing  $\approx 50\%$  non-homologous sequence. Screening of the cDNA library would be greatly facilitated by a homologous probe. Restriction of the intron from such a fragment was problematic due to a lack of suitable restriction sites and the small size of the fragments involved (between 100 and 200 bases). RT-PCR (reverse transcription-polymerase chain reaction) was employed in order to overcome this problem. The advantages were two fold: a homologous cDNA fragment containing both exons (approximately 300 bases of coding sequence) could be



obtained for use as a probe; generation of a cDNA fragment by RT-PCR would demonstrate the presence of the required transcript within the general mRNA population.

Total RNA was extracted from *Cx. molestus* pupae since late embryonic and pupal stages have been shown in *Drosophila* to be the stages of maximum AChE production (Dewhurst et al, 1970).

A cDNA fragment was successfully obtained by RT-PCR and subsequent sequence analysis revealed it as exons 2 and 3 of the AChE gene. This fragment would be a suitable probe for the isolation of a full length cDNA from a library of *Cx. molestus*. Attempts were also made using oligo dT and the primers for exons 2 and 3 to obtain a much larger fragment for use as an alternative probe and to investigate the feasibility of subsequently using RACE-PCR (rapid amplification of cDNA 5' ends-polymerase chain reaction) to obtain the 5' end of the gene.

## 7.2. Materials and Methods

Extraction of total RNA from *Cx. molestus* pupae and subsequent purification of mRNA were as described (2.32.1 and 2.32.3).

Primers for RT-PCR were designed based on the partial genomic sequence available for the *Cx. pipiens* AChE gene. Three sets of homologous primers each 20 bases long were synthesized (Genosys): KS<sub>1</sub> (specific for a stretch of sequence in exon 2) and KS<sub>2</sub> and KS<sub>3</sub> (specific for sequences in exon 3). KS<sub>1</sub> and KS<sub>3</sub> were both 5' primers and KS<sub>2</sub> the 3' (Figure 7.1).

Reverse transcription and subsequent PCR were performed as detailed (2.32.4). PCR reactions were set up at a range of MgCl<sub>2</sub> concentrations. PCR was also performed using oligo dT

Figure 7.1. Partial genomic sequence of the *Cx. pipiens* AChE gene showing exons 2 and 3 (Malcolm, pers. comm., 1994) and the positions of the oligonucleotides used as primers in the RT-PCR.



intron 1

Y E Y F P G F A G E E M W  
...TTCTTTCTTCAGATATGAATATTTCCCGGGTTTTGCCGGCGAGGAAATGTGG  
primer KS<sub>1</sub> 5'GG

N P K T N V S E D C L Y L N I W V P T  
AATCCCAAGACAAACGTATCGGAGGACTGTCTGTATCTGAACATTTGGGTACCAACGA  
AATCCCAAGACAAACGTA3'

K T R L R H G R G L N F G N N D  
AAAACCCGTTTGCGCCACGGACGAGGACTAACTTTGGAAACAACGACGTATGTACT

intron 2

TCTTCTTCTTGTTAAGTACACAGTACGACAGAAAAATGTCGATGATGGCTCTGTCAG

AGTTTTTGACAATGAGTTTTGATTCTTCGATCATTCGGAAGATTATAGTGATATGGT

GGAACGCAGTATACAGATATGAGAACTAACTTACATTTCTGTCGAGCTGTGC

TGTGATGATTTAGTTGTTTCGTGGCTCTGAGAGAGACAGGTCCAGAGTGTATTTTATAG

TAGTTGCGTAGGCGTTTATGCACCCACAACGGAGATAATTCACAAGGTTTTTTTTCT

TTTCTTTTTTGTTTTTTCCCTCTGGCATGCCGTGGCCACCTCTTTATTGCAGTACTT  
primer KS<sub>2</sub> 5'TT

Q D D D D F Q R Q H Q S K G G L A M L  
CCAGGACGATGATGACTTCCAGCGGCAGCACCAGTCCAAGGGCGGCCTCGCGATGCT  
CCAGGACGATGATGACTT3'

V W I Y G G G F M S G T S T L D V Y N  
GGTCTGGATCTACGGGGGTGGGTTTATGAGCGGAACATCAACGTTGGACGTTTACAA

A E I L A A V N V I V A S M Q Y R V G  
CGCAGAAATACTGGCGGCCGTGAACGTAATCGTGGCCTCGATGCAGTACCGAGTGGG

A F G F F Y L S P Y L N G R E E E A P  
AGCATTCGGTTTCTTCTACCTTTCGCCCTACTTGAACGGCCGTGAAGAGGAGGCTCC  
primer KS<sub>3</sub> 3'CTTGCCGGCACTTCTCCTCC5'

intron 3

AGGTGGGATAATGATGGCGATGTTTTGATCTT....

as the 3' primer. Dimethylsulphoxide (DMSO) when included in the reaction mix was at a concentration of 5% or 10% v/v.

### 7.3. Results

#### 7.3.1. Isolation of *Cx. molestus* Total RNA

A non-denaturing 1.2% w/v TBE agarose gel was used to assess the quality of RNA extracted from *Cx. molestus* (Figure 7.2.a). Ribosomal bands can clearly be seen.

#### 7.3.2. Purification of Poly A+ RNA

Purification of poly A+ RNA from total RNA was performed using Amersham messenger affinity paper (Figure 7.2.b). The equal loading of purified RNA and total RNA shows the enrichment of poly A+ species in Lane 1. Samples of this purified mRNA were later used for RT-PCR.

#### 7.3.3. RT-PCR

RT-PCR fragments corresponding approximately to the expected size were obtained for both sets of primers (KS<sub>1</sub>/KS<sub>2</sub> and KS<sub>3</sub>/KS<sub>2</sub>). MgCl<sub>2</sub> concentration was found to be critical, fragments only being obtained for final concentrations of 3 or 4 mM (Figure 7.3.a). One of the reactions at 3mM (Lane 7) seemed not to work but a fragment was later successfully obtained from this reaction by reamplification.

An additional phenomenon was observed. Lanes 5 and 6 in Figure 7.3.a show the fragments for primers KS<sub>3</sub>/KS<sub>2</sub> which have an expected size of 230 bases and an observed size approximating to this. The fragment in lane 8 generated by primers KS<sub>1</sub>/KS<sub>2</sub> has an expected size of 330 bases but it can be seen that it has run ahead of the KS<sub>3</sub>/KS<sub>2</sub> fragments



Figure 7.2.a. *Cx. molestus* total RNA run on a non-denaturing TBE 1.2% agarose gel. Ribosomal bands can be clearly seen.

Lane 1 - 1 Kb ladder  
2 - Total RNA (12 $\mu$ g)  
3 - Total RNA (12 $\mu$ g)  
4 - Total RNA (4 $\mu$ g)  
5 - Total RNA (4 $\mu$ g)

Figure 7.2.b. *Cx. molestus* poly A+ RNA purified. Enrichment for mRNA sequences can be clearly seen (Lane 1).

Lane 1 - Poly A+ RNA (2 $\mu$ g)  
2 - Total RNA (2 $\mu$ g)

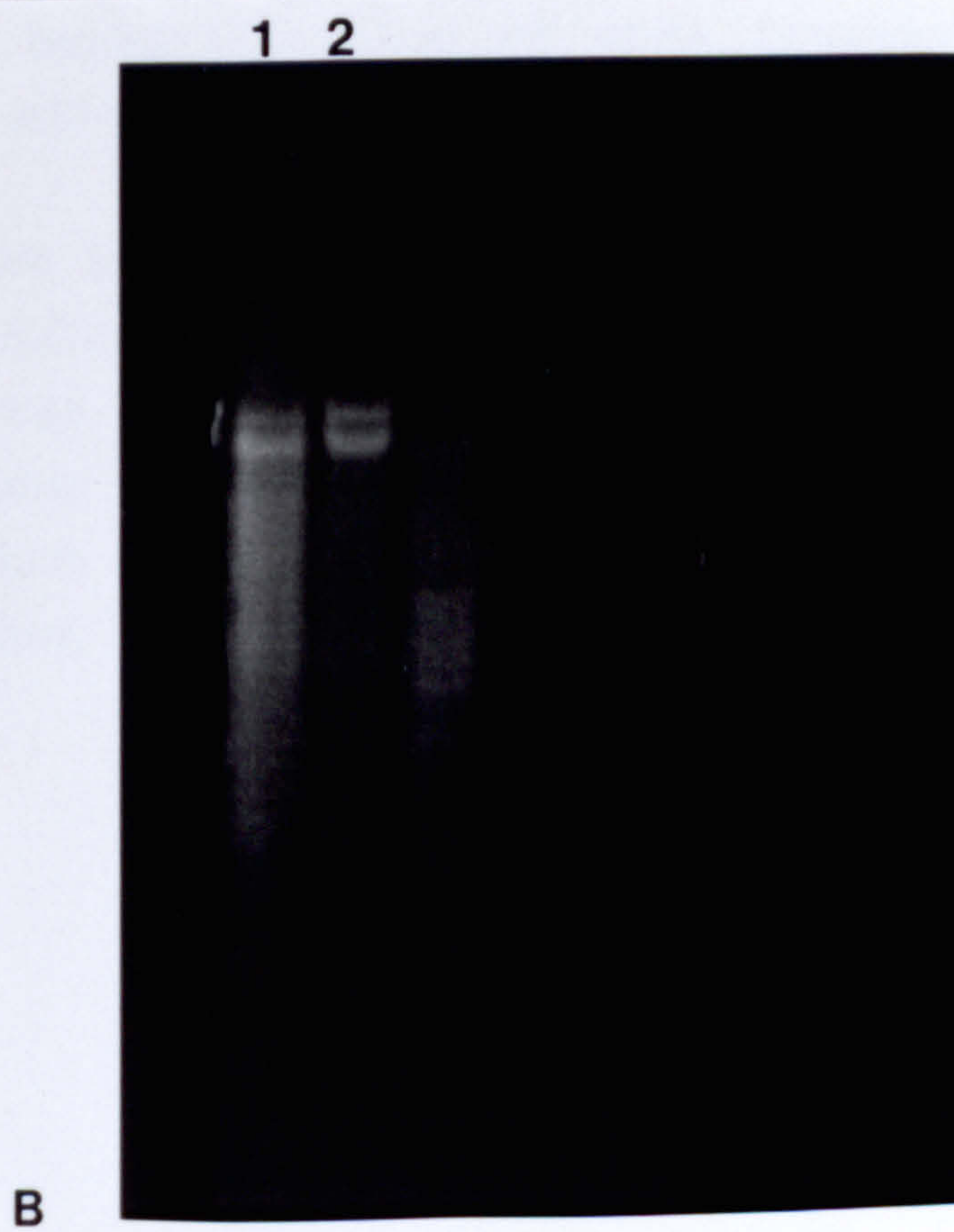
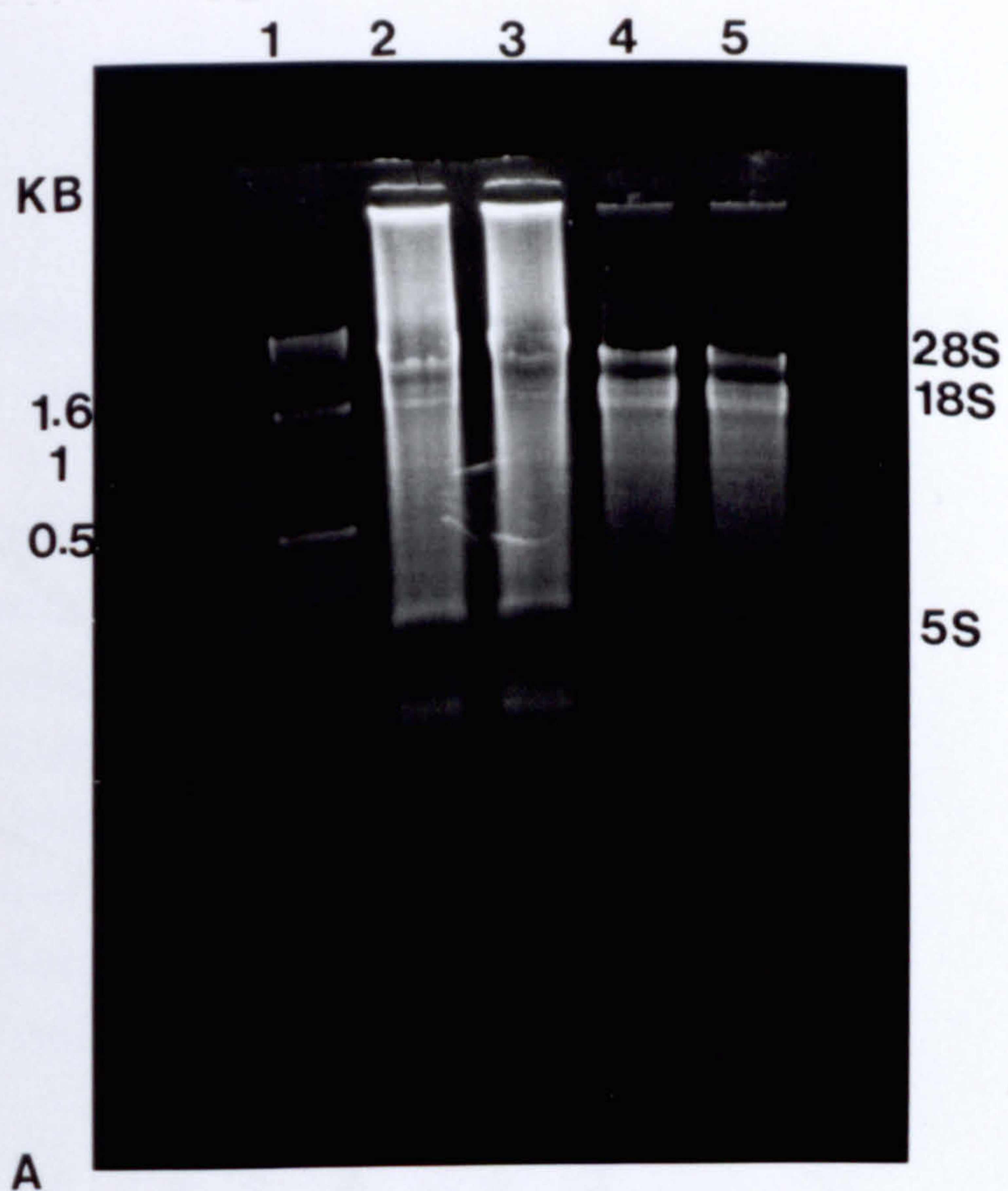




Figure 7.3.a. cDNA fragments of exon 3 and exons 2 + 3 of the *Cx. molestus* AChE gene generated by RT-PCR.

- Lane 1 - 1 Kb ladder
- 2 - Control (No DNA from RT in PCR)
- 3 - Primers KS<sub>3</sub>/KS<sub>2</sub> (1mM MgCl<sub>2</sub>)
- 4 - Primers KS<sub>3</sub>/KS<sub>2</sub> (2mM MgCl<sub>2</sub>)
- 5 - Primers KS<sub>3</sub>/KS<sub>2</sub> (3mM MgCl<sub>2</sub>)
- 6 - Primers KS<sub>3</sub>/KS<sub>2</sub> (4mM MgCl<sub>2</sub>)
- 7 - Primers KS<sub>1</sub>/KS<sub>2</sub> (3mM MgCl<sub>2</sub>)
- 8 - Primers KS<sub>1</sub>/KS<sub>2</sub> (4mM MgCl<sub>2</sub>)

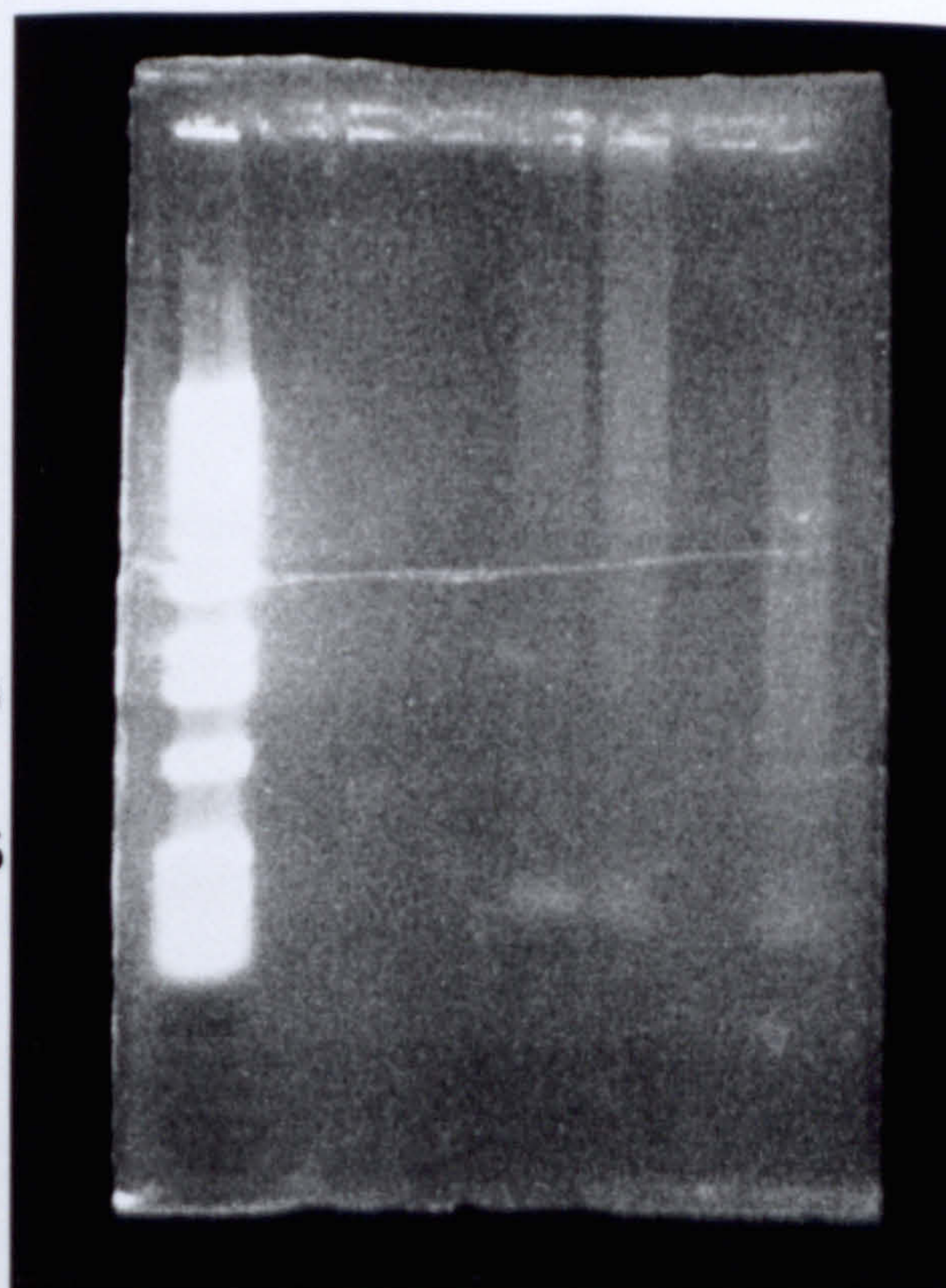
Figure 7.3.b. Reamplification of cDNA fragments at 4mM MgCl<sub>2</sub> using 2μl aliquots from reactions 5 - 8 above.

- Lane 1 - 1kb Ladder
- 2 - Control (No DNA in PCR)
- 3 - Exon 3 (Reaction 5)
- 4 - Exon 3 (Reaction 6)
- 5 - Exon 2 + 3 (Reaction 7)
- 6 - Exon 2 + 3 (Reaction 8)



1 2 3 4 5 6 7 8

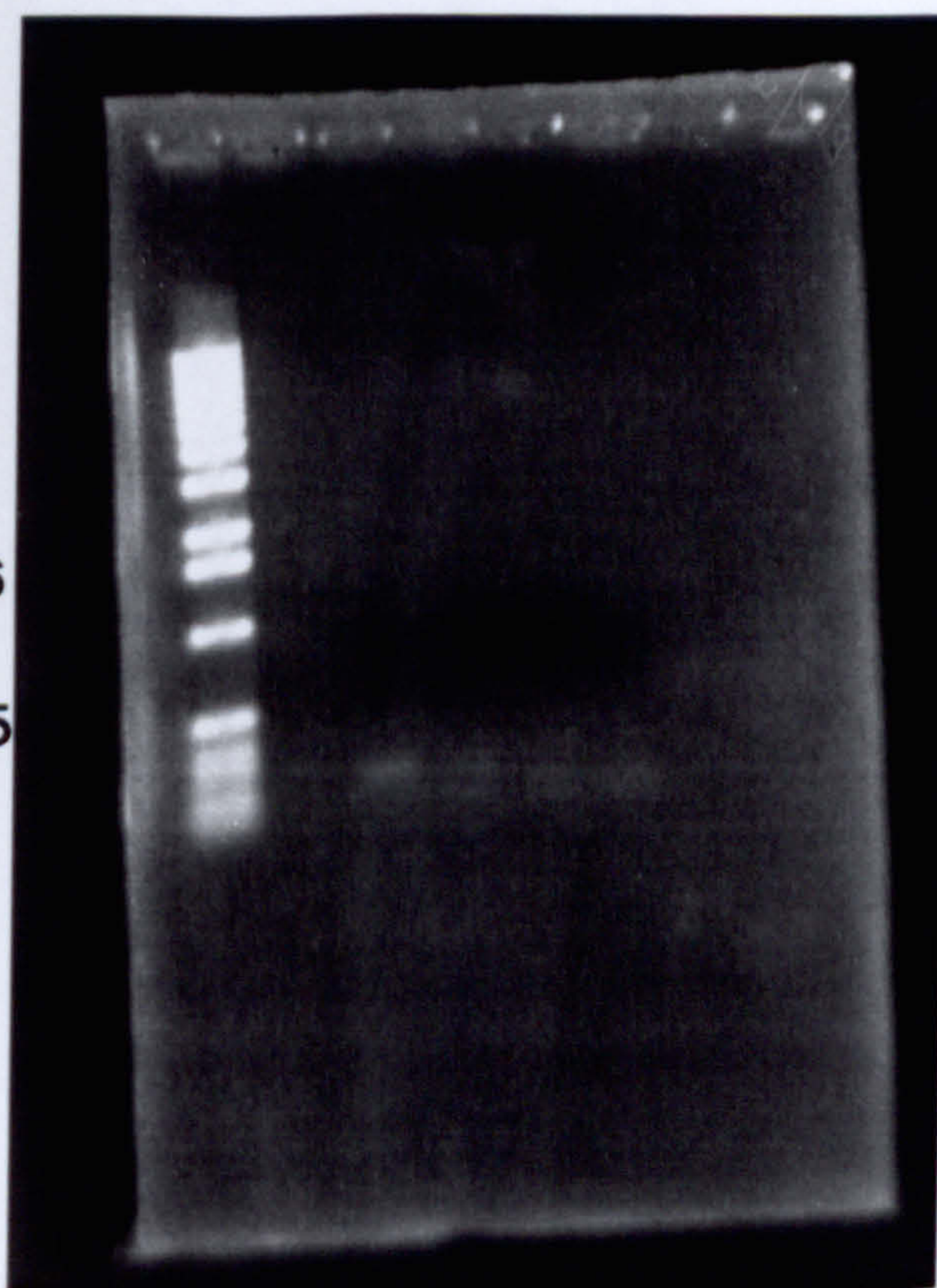
KB  
12  
3  
2  
1.6  
1  
0.5



A

1 2 3 4 5 6

KB  
12  
3  
2  
1.6  
1  
0.5



B



indicating a smaller size.

Using 2 $\mu$ l aliquots from the reactions above all four fragments were successfully reamplified (Figure 7.3.b). The KS<sub>1</sub>/KS<sub>2</sub> fragments still ran ahead of the KS<sub>3</sub>/KS<sub>2</sub> fragments.

#### 7.3.4. Reamplification with Alternative 5' Primers

Reamplifications were performed using the alternative 5' primer from the one initially used in the generation of the original fragment, to assess fidelity. Thus it was expected that primers KS<sub>3</sub>/KS<sub>2</sub> would reamplify from a KS<sub>1</sub>/KS<sub>2</sub> fragment because the latter should contain sequence homology with KS<sub>3</sub> (Figure 7.1). In contrast no amplification was expected using KS<sub>1</sub>/KS<sub>2</sub> primers on the shorter KS<sub>3</sub>/KS<sub>2</sub> fragment. Figure 7.4 shows the results of this experiment and it can be seen that amplification only occurred as expected. Lanes 5 and 6 show the shorter KS<sub>3</sub>/KS<sub>2</sub> fragment generated from the longer KS<sub>1</sub>/KS<sub>2</sub> fragment. The reverse of this could not occur due to the absence of 5' homology for KS<sub>1</sub> (lanes 3 and 4).

These results indicated that the fragments obtained were the ones expected and that the observed phenomenon of the larger fragment running further than the smaller on agarose gels was due to other factors probably related to secondary structure. Sequence analysis of the fragments KS<sub>1</sub>/KS<sub>2</sub> and KS<sub>3</sub>/KS<sub>2</sub> revealed them to correspond exactly to exons 2 + 3 and exon 3 respectively of the *Cx. pipiens* AChE gene (Malcolm pers. comm., 1995).

Several attempts were made using a variety of PCR cycling patterns (including longer extension times than used previously) to obtain a much longer cDNA fragment using oligo dT as the 3' primer but reactions failed to generate any fragments. Inclusion of DMSO at 5 and 10% v/v which

Figure 7.4. Reamplification with 'switched' 5' primers. Two fragments were obtained using  $KS_2/KS_3$  on the longer  $KS_1/KS_2$  fragment (Lanes 5 and 6). Reamplification could not occur from the shorter  $KS_2/KS_3$  fragment with  $KS_1/KS_2$  (Lanes 3 and 4).

- Lane 1 - 1Kb Ladder  
2 - Control (no DNA in PCR reaction)  
3 -  $KS_1/KS_2$  Primers and  $KS_3/KS_2$  Fragment  
4 -  $KS_1/KS_2$  Primers and  $KS_3/KS_2$  Fragment  
5 -  $KS_3/KS_2$  Primers and  $KS_1/KS_2$  Fragment  
6 -  $KS_3/KS_2$  Primers and  $KS_1/KS_2$  Fragment



1 2 3 4 5 6

KB

12

4

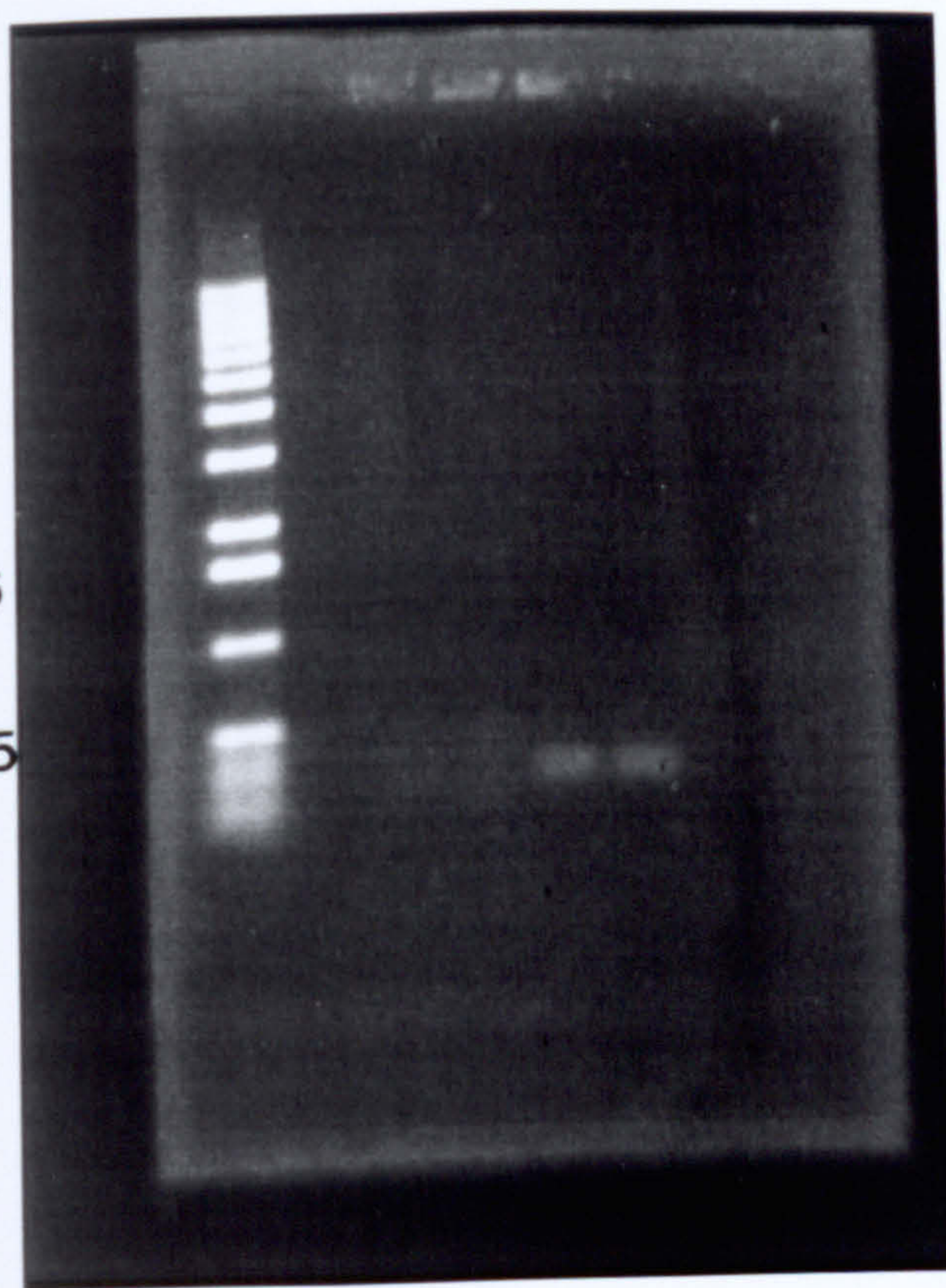
3

2

1.6

1

0.5





has been reported as improving PCR amplification of DNA with complex secondary structure (Shen and Hohn, 1992) made no difference.

#### 7.4. Discussion

cDNA fragments containing coding sequences corresponding to exons 2 + 3 and exon 3 of the *Cx. pipiens* AChE gene were produced using RT-PCR from purified *Cx. molestus* mRNA. Either of these fragments could be used as a suitable homologous probe for the screening of a cDNA library from *Cx. molestus* to obtain a susceptible mosquito AChE cDNA.

The success of the reverse transcription, which used oligo dT as the primer, suggests that the mRNA obtained was of good quality and that the transcript is likely to be of full length. Evidence for the latter assertion stems from the fact that the primers used for the PCR are towards the 5' end of the gene (exons 2 and 3).

An alternative, and quicker, approach to the obtaining of the full length cDNA would be the use of RACE-PCR. However, attempts to generate a longer cDNA fragment from the 3' end using oligo dT and KS<sub>1</sub> or KS<sub>2</sub> as primers were unsuccessful. The reason for this remains unclear, since aliquots from the original reverse transcription reactions, from which the shorter PCR fragments had been obtained, were used throughout.

A disadvantage of using PCR to obtain the full cDNA for use in future expression studies is that the number of sequence errors is likely to be higher than if a cDNA clone is isolated from a library. However, since sequencing of a cDNA derived from either method would be required and comparison made with existing genomic sequences of mosquito AChE genes the level of error in a PCR derived cDNA could be accurately assessed.



## CHAPTER EIGHT GENERAL DISCUSSION

Biologically active AChE from *D. melanogaster* was successfully expressed in the yeast *S. cerevisiae* under the constitutive *GPD* promoter using plasmid pG3 in the protease deficient strain BJ2168. On extraction from protoplasts the amounts of active enzyme were found to be at a level well within the range of detection by the method of Ellman *et al* (1961) (5.3.4). Effectively for the purposes of the future examination of AChE variants an expression system in *S. cerevisiae* has been shown to be feasible since the recombinant enzyme was successfully inhibited with both the insecticide Bendiocarb (5.3.5) and the cholinesterase inhibitor PMSF (6.3.3) and the effects of inhibition could be measured kinetically using a microtitre plate reader. The cell wall interference by *S. cerevisiae* noted in the Ellman assay (3.3.2) although undesirable has been shown to be far from prohibitive when protoplasts are used (5.3.4; 5.3.5; 6.3.3). The  $K_{cat}$  value of the expressed DAChE was calculated at a value approaching that reported by Taylor *et al* (1974) for purified AChE from electric ray (6.3.3) and similar to that reported by Kronman *et al* (1994) for human AChE. Results of the inhibition studies and SDS-PAGE analyses were indicative of low levels of biologically active enzyme in the correct conformation.

AChE has been expressed in several different heterologous systems: *Xenopus* oocytes (Fournier *et al*, 1992; Soreq *et al*, 1990), mammalian cells (Gibney *et al*, 1990; Velan *et al*, 1991a), baculovirus systems (Radic *et al*, 1992; Chaabihi *et al*, 1994; Anthony *et al*, 1995), and *E. coli* (Fischer *et al*, 1993; Fischer *et al*, 1995) and of particular relevance in the yeast *P. pastoris* (Morel and Massoulie, 1997) but here is described the first successful expression in *S. cerevisiae*.

### 8.1. Comparative Levels of Recombinant AChE Expression

Expression levels of *inactive* human AChE in the alternative microbial system of *E. coli* have been shown to reach as much as 10% of total bacterial proteins and this quantity is easily visible as a separate intense band on Coomassie stained polyacrylamide gels (Fischer et al, 1993). The word 'inactive' is important. The heterologous AChE described in *E. coli* required solubilization and refolding to achieve activity because the expressed enzyme was found to be embedded in inclusion bodies. Biologically active *D. melanogaster* AChE expressed in *S. cerevisiae* can be easily liberated from lysed protoplasts without the need for solubilization and refolding and this immediately makes it a more desirable system than *E. coli* for the long term aim of examining insecticide resistant mutant AChEs. Interestingly, the *E. coli* enzyme became significantly more active following substitution of the seventh cysteine 580 residue which is thought to be involved in dimerization (Velan et al, 1991). This much higher activity was a reflection of a 40 fold increase in refolding yields of the inactive form of the enzyme (Fischer et al, 1995). The *Drosophila* enzyme contains an even number of cysteine residues (8), six involved in the well documented intrachain disulphide bonds of AChE, one unpaired (Cys<sup>328</sup>), and one (Cys<sup>615</sup>) thought to be involved in the formation of the native dimer, much as Cys<sup>580</sup> is in the human molecule (Mutero and Fournier, 1992). It seems unlikely that the DAChE from *S. cerevisiae* is incorrectly folded (at least in terms of conformation necessary for activity), despite the presence of Cys<sup>328</sup> and Cys<sup>615</sup> (although Cys<sup>615</sup> would not be present in a product truncated from the 3' end see below), however, because of the absence of large quantities of inactive protein and the efficiency of substrate hydrolysis as evidenced by the  $K_{cat}$  results.

The refolding efficiency *in vitro* of the human AChE from *E.*



*coli* is 'below' 0.1% (Fischer et al, 1995). A 40 fold increase in refolding yield for the mutant enzyme containing the Cys<sup>580</sup> substitution, assuming a yield of 0.1% for the wild type human AChE, results in a yield of  $\approx 4\%$  of a total inclusion body percentage of 44%. Thus the percentage of correctly folded active AChE from *E. coli* was 1.76% for a reported activity of 46100 nmoles substrate/min/mg protein (Fischer et al, 1993). The ratio of activity to amount of biologically active enzyme was therefore 26193.2:1. A specific activity of  $\approx$  or  $>50$  Units was routinely obtained for the DACHe from *S. cerevisiae* for a percentage of total protein of 0.0009%. This results in a ratio of 55555.6:1 - within a factor of 2 of that calculated for the *E. coli* AChE.

For the purposes of the analysis of insecticide resistant variants a yeast system has the same ease and convenience that expression in *E. coli* offers but with the advantage of a biologically active enzyme without the need for any post-extraction treatment. For large scale production *S. cerevisiae* is safer, trusted, and cheaper than *E. coli*. The fact that a biologically active enzyme was recovered from *S. cerevisiae* points to the importance of the eukaryotic secretory pathway in the post-translational modification and processing of a complex protein such as AChE. The expressed DACHe was mainly found to be associated with the cell membrane in *S. cerevisiae* (6.3.5.a) suggesting that the recombinant protein had been processed through the secretory pathway (see below). The remainder of the activity may have been derived from a proportion of the DACHe inserted in ER or Golgi derived vesicles of the microsomal fraction or possibly from soluble free DACHe in the cytoplasm.

The low level of AChE expression found in *S. cerevisiae* correlates to a large extent with the levels of expression reported for rat and *Bungarus* AChE in *P. pastoris* using

AOX1 based integrative vectors. Morel and Massoulie (1997) report production levels of  $\approx 1\text{mg/L}$  and  $\approx 2\text{mg/L}$  for rat and *Bungarus* AChEs respectively. These levels of production are quite clearly of the order of 500X lower than has been reported for other heterologous proteins expressed in *P. pastoris* such as bovine lysozyme ( $0.55\text{g/L}$ ) (Digan et al, 1989) or recombinant tick anti-coagulant peptide ( $1.7\text{g/L}$ ) (Laroche et al, 1994). At first sight, however, production of vertebrate AChE in *P. pastoris* seems markedly higher than that reported in this study for the insect AChE from *S. cerevisiae*. Approximately 5ng of active DACHe were obtained for a 50ml culture of BJ2168R grown on MM to an  $\text{OD}_{600}$  of 0.1 (2.22.1; Appendix 3.4). This would correspond to only  $\approx 100\text{ng}$  of active AChE protein per litre of culture. However, it should be noted that the cell density of *P. pastoris* reported by Morel and Massoulie (1997) was  $5 \times 10^9$  cells/ml. The measurements of DACHe activity reported here were taken from MM cultures of *S. cerevisiae* grown to a density of  $\approx 2 \times 10^6$  cells/ml. The cell density of *S. cerevisiae* was thus approximately 2500X lower than that reported by Morel and Massoulie (1997) for *P. pastoris*. When this factor is taken into account the expression level per cell is in far closer agreement than the raw data suggest.  $100\text{ng} \times 2500 = 0.25\text{mg/L}$  of *S. cerevisiae* culture at the same cell density.

One recognised advantage of *P. pastoris* as a yeast heterologous expression system is the capability of attaining very high cell densities (Wegner, 1983). It should be noted, however, that *S. cerevisiae* can be grown to far higher cell densities on rich media (of an order of magnitude) (although lower than *P. pastoris*) and since the DACHe was shown to be expressed in stationary phase (5.3.2) harvesting at this point in the growth cycle is also a possibility to increase cell density further. The higher cell densities achievable in *P. pastoris* would need to be balanced against the long history of safe use of *S.*



*cerevisiae* in large scale fermentation particularly in the light of the therapeutic potential of a recombinant AChE for treatment of or prophylaxis against neurotoxins targeting the AChE enzyme.

What is clear is that expression levels in both yeast (although somewhat higher in *P. pastoris*) is low and that defining parameters for high level production in yeast remain to be determined. The high levels of expression of insect AChE reported in baculovirus systems are in marked contrast. Chaabihi et al (1994) reporting on the expression of DACHe from recombinant baculoviruses in *Spodoptera frugiperda* cells estimate the expression rate at approximately 1mg/L ( $10^9$  cells in total =  $10^6$  cells/ml). This is clearly of the order of 10000X the level of expression per cell than that reported here for DACHe in *S. cerevisiae*. Baculovirus systems are convenient, relatively cheap, and now are proving the system of choice for the investigation of insect AChEs (Anthony et al, 1995; Vaughan et al, 1997).

Much research has been achieved using *Xenopus* oocytes (Fournier et al, 1992) or mammalian systems such as embryonic kidney cell lines (Velan et al, 1991). These systems have the advantage, like insect cell systems, of high levels of expression per cell, for example Kronman et al (1992), report levels as high as 5-25pg of enzyme per cell for embryonal kidney 293 cell lines transfected with vectors containing cytomegalovirus, Rous sarcoma virus, or simian virus 40 promoters to drive the production of human AChE. In contrast when production levels per cell for *S. cerevisiae* based on 50ml cultures at  $2 \times 10^6$  cells/ml (resuspended in 1.5ml) are combined with the mean amount of DACHe protein/well at an assay volume of 120 $\mu$ l/well (Appendix 3.4) a value of approximately 0.00005pg/cell is obtained. Again the difference is of the order of 10000X. In the absence of a suitable and efficient microbial system

for recombinant AChE, mammalian cell culture is used by companies such as Sigma for large scale production. These mammalian systems are expensive, cumbersome, and unable to attain the enormous biomass that can be achieved in microbial cultures. Expression of DACHe in *Xenopus* results in extremely high levels of expression per oocyte (4-5 orders of magnitude higher/cell than *S. cerevisiae*) (Fournier et al, 1992) but obviously such a system is completely incompatible with scale up and will remain a powerful laboratory research tool. Baculovirus systems are more convenient but once again do not possess the advantage of the very high cell densities and ease of large scale bulk production associated with yeast systems. Furthermore the long term safety of recombinant human proteins produced in baculovirus systems has yet to be elucidated. Given that biologically active AChEs, with early indications of correct conformation, have now been expressed in both *S. cerevisiae* and *P. pastoris* focusing of future research in increasing levels of production using alternative vectors, alternative yeasts, may yield a superior eukaryotic microbial production system for AChE.

## 8.2. Protein Processing

In addition to the high level of expression attained in *Spodoptera* cells the recombinant *Drosophila* AChE was shown to be completely processed by the insect secretory system including cleavage of the signal peptide, cleavage of the precursor, formation of functional dimers and the attachment of a glycosylphosphatidylinositol (GPI) anchor (Chaabhi, 1994). This is perhaps not so surprising in this case since DACHe is an insect protein being expressed in insect cells. The precise molecular recognition required for the correct and efficient processing (and subsequent secretion) of a complex heterologous protein might be predicted to occur when the evolutionary distance between the source of the protein and the host cell is particularly



close. Similar results may come to light when the recombinant *Ae. aegypti* AChE is further characterised in the baculovirus system (Anthony et al, 1995).

In *Xenopus* oocytes a difference in processing of DACHe is seen. Intracellularly expressed *Xenopus* DACHe containing the C-terminal region is always associated with the microsomal particulate fraction of homogenised oocytes suggesting that the protein is retained in subcellular compartments such as the ER. The hypothesis follows that the expressed AChE is directed through the secretory pathway in *Xenopus* (the signal peptide of preAChE is cleaved away) but that *Xenopus* does not recognise the signal for replacement of the C-terminus with a GPI anchor and that interaction between the retained C-terminus and cellular compartments leads to 'trapping' of the recombinant DACHe in the secretory pathway. This is supported by three further pieces of evidence: one that the C-terminus is still retained by the partially mature enzyme in *Xenopus* (shown by binding of specific antibody against it), secondly that expression of a cDNA truncated for the C-terminus results in complete secretion and release of the DACHe to the culture medium and thirdly the expressed full length DACHe is insensitive to phosphatidylinositol phospholipase C (PLPC) (Fournier et al, 1992). Additionally, the *Xenopus* complete DACHe polypeptide is not fully matured since cleavage at the hydrophilic sequence into the two non-covalently associated polypeptides of 55 and 18 KDa does not occur. This uncleaved partly matured DACHe is still enzymatically active. Interestingly, secretion of the protein truncated for the last 27 amino acids of the C-terminus results in cleavage at the hydrophilic site and full maturation (apart from addition of the anchor) (Fournier et al, 1992). Full maturation is thus linked to efficient secretion, in *Xenopus* at least. How, then, is the recombinant DACHe enzyme processed by *S. cerevisiae*?

Certain indications of the processing of the recombinant DACH<sub>E</sub> at the RNA and protein level by *S. cerevisiae* can be gleaned from the results at hand. The significant contribution of an unstable mRNA shown by northern analysis of total RNA isolated from DACH<sub>E</sub> expressing *S. cerevisiae* cultures to the low levels of expression observed has already been discussed (6.4). A putative enzymatically active truncated protein translated from the shortened transcript shown by the northern analysis has also been mentioned and the comprehensive nature of the SDS-PAGE analysis and its failure to highlight the recombinant DACH<sub>E</sub> discussed (6.4). However, processing of such a truncated translation product by *S. cerevisiae* or further possibilities remain to be examined.

It is possible that the low level of shortened DACH<sub>E</sub> transcript detected on the northern blot is not the transcript responsible for the active enzyme found associated with the cell membrane fraction. Very low levels of a full length transcript undetectable by northern blotting of total RNA may be translated into a full length DACH<sub>E</sub> polypeptide that is the protein detected by activity assay. A high degree of instability of the DACH<sub>E</sub> transcript seems likely but the precise mechanisms determining mRNA stability are only beginning to be understood. mRNA processing between yeasts is known to differ, for example, expression of a 30 KDa *Candida albicans* membrane protein gene in *S. cerevisiae* can be detected as three different sized transcripts whereas in the native host only the one full length transcript is seen. The full length transcript of 1.1 Kb is seen in *S. cerevisiae* and presumably leads to the 30 KDa protein product that is observed but two other transcripts of 0.7 and 0.5 Kb are also observed and do not appear to be translated (Iborra et al, 1996). It is possible that these are 'stalled' degradation products or mistranscribed



sequences due to differences in mRNA processing between two species that are close in terms of evolution.

Degradation proceeding from either the 5' or the 3' end of the DAcHE transcript is possible. In either case the detection of the 1.7 Kb transcript on the northern blot (Figure 6.5) albeit at a low level argues for some degree of stability at this point in the degradative process. Whether the pause or 'stalling' in ribonuclease activity at this sequence location leads to a transcript stable enough for translation is purely speculative. Only degradation from the 3' end is compatible with the possibility of an enzymatically active polypeptide product (6.4). Degradation from the 5' end remains possible and indeed there is recent evidence that the action of two *S. cerevisiae* 5' exoribonucleases XRN1 and HKE1 can be stalled at any point in the transcript length by the occurrence of secondary structures (Poole and Stevens, 1997). If this were the case then the 1.7 Kb transcript is merely a transiently stalled degradation product and not translated. The active DAcHE detected could then be envisaged as the translated product of a small population of full length transcripts that effectively 'escape' the strong tendency of this heterologous transcript to be rapidly degraded by the *S. cerevisiae* mRNA processing machinery. Morel and Massoulie (1997) do not include an analysis of transcription levels of the *P. pastoris* recombinant vertebrate AChE but it would be interesting to see whether the relatively low level of expression in *Pichia* was due to a putative unstable RNA or whether large quantities of transcript of the expected length would be observed, indicating translational or protein degradative problems. This is especially salient in that Morel and Massoulie (1997) suggest that there is no evidence for 'the massive production of inactive rat or *Bungarus* AChE production in the culture medium'. This is similar to the situation for DAcHE in *S. cerevisiae* evidenced by the SDS-PAGE results



presented here (6.3.4; 6.3.5.b) and in marked contrast to AChE expression in *E. coli* (Fischer et al, 1993; Fischer et al, 1995). However, it should be mentioned that Morel and Massoulie (1997) do not present any SDS-PAGE or Western blotting results (even though it was used to correlate the amount of protein with activity levels using different constructs) and further that only the concentrated culture medium was examined. It is possible that inactive misfolded protein would not be secreted into the medium but would remain associated with the cells even for the recombinant *Bungarus* AChE in which  $\approx 90\%$  of the active enzyme was secreted into the medium. The cellular fraction would also need to be investigated. It remains possible then, although unlikely, that large amounts of inactive protein are present for the recombinant vertebrate AChEs when expressed in *P. pastoris*.

With regard to the secretory pathway in *S. cerevisiae* processing of two putative DACHe transcription products needs to be considered. The postulated location of the DACHe in the cell membrane is indicative of transport through the secretory pathway of *S. cerevisiae*. It is reasonable to assume that the native signal peptide is recognised and sufficient for translocation of the translated product to the ER. Recognition and processing of the native signal peptide has been shown to be sufficient for the secretion of human interferons for example (Hitzeman et al, 1983) or for the secretion of human  $\alpha$ -amylase (Sato et al, 1986). Processing of the heterologous signal peptide may occur at the correct cleavage site by the yeast signal peptidase or some misprocessing may occur to a greater or lesser extent. Alternatively only a proportion of the heterologous protein may be processed at the signal peptidase level at all leading to intracellular accumulation of a large percentage of the product. This occurred when human growth hormone was secreted in *S. cerevisiae* by means of its native



presequence: 90% of the protein was found to retain the entire secretion signal and was associated with the cells; the 10% that was successfully secreted, however, was found to be correctly processed (Hitzeman et al, 1990).

Vertebrate AChE has been shown to be directed through the secretory pathway by *P. pastoris* on the basis of its native signal peptide (Morel and Massoulie, 1997). Interestingly, yields were lower when a homologous signal peptide such as the  $\alpha$ -factor was used. Further, the  $\alpha$ -factor preprosequence was more efficient than the  $\alpha$ -factor presequence alone, despite the fact that native AChE does not contain a prosequence; but when the  $\alpha$ -factor prosequence was used in conjunction with the native presequence levels were still lower than with the native presequence alone (Morel and Massoulie, 1997). It would be interesting to examine whether this was the case with expression of AChE in *S. cerevisiae*. Whether the signal peptide of preAChE is efficiently cleaved on translocation to the ER by either yeast and if so at which exact site remains to be demonstrated; but the fact that intracellular accumulation of DACHe by *S. cerevisiae* was not observed in the current study and that the 'end of the line' for the secretion of the DACHe enzyme on the basis of its native signal peptide in *S. cerevisiae* seems to be the cell membrane would suggest that signal peptidase cleavage occurs. Proteins in which signal peptidase cleavage has been disrupted in *S. cerevisiae* have a tendency to accumulate intracellularly within the ER (Schauer et al, 1985; Haguenauer-Tsapis et al, 1986; Bohni et al, 1988). This also would appear to be the case for the full length *P. pastoris* rat AChE which was found mainly associated with the cell wall and had thus not accumulated intracellularly. There appears to be an interesting difference between the processing of the recombinant DACHe and the rat AChE by the two yeasts at this late stage. Morel and Massoulie (1997) note that substrate hydrolysis occurred at approximately

the same rate by intact cells or mechanically ruptured cells and suggest that the enzyme is trapped in the periplasmic space. However, hydrolysis by intact cells is hard to reconcile with the presence of the enzyme in the periplasmic space, rather it suggests that the enzyme is secreted (including its active site) through the cell wall but not released into the medium. This is in contrast to the putative location of the recombinant DChE active site internal of the cell membrane of *S. cerevisiae*. Results presented here show that substrate hydrolysis did not occur by either intact protoplasts or the protoplasting supernatant which would have been expected if the DChE were trapped in the cell wall, released into the periplasmic space or the active site was external to the plasma membrane (6.3.5.c; 6.3.5.d).

Results based on sedimentation co-efficients for the molecular forms of the recombinant AChEs produced in *P. pastoris* suggest that full length AChE polypeptides are produced and processed into the same forms by *P. pastoris* as *in vivo* (cleavage at a hydrophilic central sequence into two polypeptides does not occur in vertebrate AChEs). There is no evidence for the presence of a truncated product when AChE is expressed in *P. pastoris*. Recognition of the rat C-terminal GPI addition signal (present in the alternatively spliced 'H' domain of vertebrate AChEs (Velan *et al*, 1991)) by *P. pastoris* also seems to occur with replacement of the C-terminus with a GPI anchor (Morel and Massoulie, 1997). This is a significant finding since the *Drosophila* addition signal is not recognised by *Xenopus* (Fournier *et al*, 1992).

If the active DChE observed in the cell membrane of *S. cerevisiae* is truncated then the C-terminus would not be present and there would obviously be no replacement by a GPI anchor. It is perhaps difficult to reconcile such a severely truncated protein with the efficient enzyme



demonstrated by the calculation of  $K_{cat}$ , despite the presence of the active site and residues shown to be important for catalysis in such a putative translated polypeptide (6.4). Furthermore, transport through the secretory pathway as far as the cell membrane may indicate that the activity observed is due to a correctly processed full length AChE since a correct tertiary and quaternary structure is usually required for transport competence out of the ER (Schonberger et al, 1996). There is evidence that misfolding of proteins particularly in relation to crucial Cys disulphide linkages results in intracellular accumulation and concomitant poor secretion. For example when bovine pancreatic trypsin inhibitor (BPTI) expressed in *S. cerevisiae* was subjected to site-directed mutagenesis of Cys residues so that the influence of disulphide bonds at particular sites on secretion of the protein could be examined it was found that certain linkages were more important than others for subsequent secretion. Disruption of one bond in particular resulted in complete inhibition of secretion (Kowalski et al, 1998). Secretion of ovine  $\beta$ -lactoglobulin ( $\beta$ -LG) into the culture medium by *S. cerevisiae* using its native secretion signal has also been shown by site-directed mutagenesis to be dependent on the correct formation of the two disulphide bonds present in  $\beta$ -LG (Rocha et al, 1996). Folding around disulphide linkages in the ER is thus correlated with secretory competence in *S. cerevisiae*. The truncated DACH polypeptide derived from a 3' degraded transcript discussed previously (6.4) would not contain Cys<sup>598</sup> and would thus not be able to form the Cys<sup>480</sup>-Cys<sup>598</sup> linkage of DACH (Mutero and Fournier, 1992). Whether this linkage would be vital to secretory competence or conformation in relation to activity remains to be discovered but it can be envisaged that misfolding due to the absence of Cys<sup>598</sup> may occur leading to retention in the ER. Since Cys<sup>615</sup> would also be absent correct dimerization of DACH monomers would also be impossible. However, again, there is no evidence of large amounts of

intracellularly retained inactive enzyme (as shown by the SDS-PAGE analysis) and the major proportion of activity was found to be associated with the cell membrane fraction.

There is the strong possibility that a misfolded and/or truncated product would be subject to recognition by the ubiquitin pathway and rapidly transported back to the cytoplasmic side of the ER for degradation (Hershko et al, 1981; Hiller et al, 1996). If this is not the case then the transport of an active truncated DACH<sub>E</sub> product to the cell membrane is extremely interesting. Assuming cleavage of the signal peptide within the ER and absence of the C-terminus then a truncated DACH<sub>E</sub> may locate in the cell membrane by virtue of its central hydrophobic portion although it should be noted that this region is preceded by a hydrophilic region and hydrophobicity may not be sufficient to locate as a transmembrane segment (Hall and Spierer, 1986). It is possible, of course, that the signal peptide is retained or misprocessed upstream of the natural cleavage site such that a hydrophobic portion is retained. A degree of misprocessing of the IFN- $\alpha$ 1 native presequence by *S. cerevisiae* has been reported that occurred  $\approx$  15 amino acids upstream of the correct site (Hitzeman et al, 1983). Insertion of the N-terminus in the membrane may explain the observed location of the active site inside the cell rather than externalised.

An alternative hypothesis is that the active DACH<sub>E</sub> is representative of a proportion of full length transcripts that have been translated and duly directed through the secretory pathway. Again the presence of a significant proportion of the enzyme in the cell membrane argues for secretory competence of the expressed DACH<sub>E</sub> by virtue of its native signal sequence. The efficiency of substrate hydrolysis as indicated by the  $K_{cat}$  is suggestive of a native conformation and that formation of and correct folding around the three intrachain disulphide bonds (Mutero and



Fournier, 1992) has probably occurred enabling transport from the ER to the cis-Golgi.

Since the SDS-PAGE analysis failed to highlight a recombinant DAcHE band examination of mobility shifts following growth of BJ2168R cultures in the presence of tunicamycin was not possible to ascertain levels of N-linked glycosylation or even whether the DAcHE is glycosylated at all by *S. cerevisiae*. Tunicamycin was used to exclude the possibility that hyperglycosylation of the DAcHE may have led to significant retardation in an SDS-PAGE gel and that such a modified protein may not have been covered by the analysis. This has already been discussed (6.4). N-linked hyperglycosylation of recombinant proteins by *S. cerevisiae* is well documented (Moir and Dumais, 1987; Hitzeman et al, 1990; Cummings and Fowler, 1996; Kang et al, 1996; Ribo et al, 1996) and this can sometimes lead to problems in terms of activity or immunogenicity problems. It should be remembered that 'hyperglycosylation' refers to the additional outer chain mannose modifications often conferred upon heterologous proteins by *S. cerevisiae* during protein processing. This additional glycosylation is not 'hyper' in terms of recognition and subsequent processing by the yeast cell however, since homologous secretory proteins are modified in this way. In other words hyperglycosylation may not affect secretory competence as evidenced by the full secretion of such hyperglycosylated products.

DAcHE has five potential sites of N-linked glycosylation but only four are actually glycosylated by *Xenopus* (Hall and Spierer, 1986; Mutero and Fournier, 1992) and this possibly mimics the situation *in vivo*. Glycosylation of the protein is not necessary for catalytic activity, indeed measurements of  $K_m$  values are similar whether DAcHE expressed in *Xenopus* is glycosylated or not. Furthermore, Mutero and Fournier (1992) demonstrate that glycosylation

is not necessary for secretion from the oocyte. Secretion of a protein by *S. cerevisiae* is also not necessarily dependent on homologous glycosylation patterns as the successful secretion of proteins modified by site-directed mutagenesis or in glycosylation mutants such as *mmn9* have shown (Myajima et al, 1986; Moir and Dumais, 1987; Melnick et al, 1990). Thus glycosylation of the DACH<sub>E</sub> by *S. cerevisiae* may not be necessary either for activity or for export to the cell membrane. It may, however, be related to the trafficking efficiency with which the protein is secreted since glycosylation is known to play a role in the rate of protein folding and secretory transport from the ER in *S. cerevisiae* (Winther et al, 1991; Riederer and Hinnen, 1991; Holst et al 1996). It is impossible to say whether the *S. cerevisiae* DACH<sub>E</sub> is glycosylated and to what extent but it seems reasonable to assume that at least some degree of glycosylation would take place, facilitating adoption of a correct conformation and subsequent transport to the cell membrane. The passage of the DACH<sub>E</sub> through the Golgi en route may also lead to addition of outer chain modifications to any of the five potential sites (the putative truncated product would lack Asn<sup>569</sup> but this is also the site that is not glycosylated by *Xenopus* (Mutero and Fournier, 1992)).

It remains to be seen whether the hydrophobic C-terminus of a full length DACH<sub>E</sub> would be replaced by a GPI anchor in *S. cerevisiae*. It would appear at least if the activity observable in the cell membrane fraction is due to a full length product that the C-terminus does not act as an ER retention signal as has been suggested to be the case in *Xenopus*. The difference in the observations of activity associated with intact cells in the case of recombinant AChE in *P. pastoris* and only with ruptured protoplasts in the case of DACH<sub>E</sub> in *S. cerevisiae* suggests a significant difference with regard to anchorage in the cell membrane. Possible retention of the hydrophobic C-terminus may in



some way affect conformation such that the protein is threaded back through the phospholipid bilayer either from the luminal side of the ER membrane or the lumen of derived vesicles during transport so that subsequent fusion with the plasma membrane effectively places the active site on the cytosolic side.

### 8.3. Vector Choice and Suggestions for Further Work

Efficient secretion of the DAcHE to the culture medium was not the immediate goal using the constitutive episomal vector pG3. However, by retaining the native secretion signal of DAcHE, processing through the secretory pathway was a possibility. The evidence presented here suggests that this is indeed the case and that a correctly processed and active enzyme is transported to the cell membrane of *S. cerevisiae*. There remains also the possibility that a processed truncated form of DAcHE in *S. cerevisiae* can adopt a conformation around a retained active site that is conducive to high catalytic activity. The major obstacle therefore, does not appear to relate to processing of the protein through the secretory pathway but to the general level of expression.

Expression from construct pBM150-DAcHE either did not occur or was at undetectable levels. Despite the fact that pBM150 is a single copy plasmid it contains the well characterised GAL promoter which is induced on galactose by about 2000 fold (Kingsman et al, 1990). Numerous examples of the successful production of heterologous proteins using the GAL promoter exist (Goff et al, 1984; Roy et al, 1995; Nomura et al, 1995). pBM150 itself has been used for the successful expression of heavy and light chain immunoglobulins (Carlson, 1988), however expression of DAcHE using pBM150 and the GAL10 promoter was unsuccessful. The fact that subsequently detectable levels of expression were achieved, albeit at a low level, using a high copy

number plasmid and a suitably strong promoter *GPD* suggests that copy number may be important. It may be possible to increase levels of expression by raising copy number still further for example by the use of an *leu-2d* marker. Such a system which can raise plasmid copy number levels to as much as 400-500 using the entire 2 $\mu$  form B plasmid in conjunction with pBR322 sequences and a cir<sup>o</sup> *S. cerevisiae* strain can significantly enhance expression levels. For example, use of the *leu-2d* system in conjunction with the *GAL1* promoter for the expression of chloramphenicol acetyl transferase increased production by  $\approx$  60X and perhaps significantly, in the light of the SDS-PAGE results presented here, enabled the product to be easily visualised by SDS-PAGE whereas before using a routine episomal plasmid for expression it was undetectable (Hadfield, 1994).

The use of standard 2 $\mu$  based vectors and a powerful constitutive promoter such as *GPD* for the high level expression of heterologous proteins is well documented (Bitter and Egan, 1984; Travis et al, 1985; Kniskern et al, 1986). Expression levels using such constructs and the *GPD* promoter are noted to vary from anything between 0.1% and 30% of soluble cell protein (Rosenberg et al, 1990). Therefore, until such constructs and promoters are tried it is difficult to predict what the level of heterologous expression will be since it depends on so many additional factors.

DAC<sub>H</sub>E is a moderately complex eukaryotic protein containing both hydrophobic and hydrophilic sequences requiring processing into a conformation around the active site that is conducive to catalytic activity. Plasmid pG3 in conjunction with the *GPD* promoter has been successfully used for the expression of rat glucocorticoid receptor consisting of 795 amino acids ( $\approx$  88 KDa) which is of similar size and complexity to the 75 KDa monomer of DAC<sub>H</sub>E. Expression levels were found to be  $\approx$  0.1% of soluble



protein in BJ2168 which could be enhanced to  $\approx 1\%$  in *S. cerevisiae* strains of shorter generation (Schena et al, 1991). This suggests that using vector pG3 initial expression levels of DACHe might have been expected to be at least 10 fold if not 100 fold higher than observed. Testing of the pG3-DACHe construct in alternative strains may yield increased expression levels of DACHe.

Other similarly complex eukaryotic proteins have been and continue to be successfully expressed in *S. cerevisiae* using similar vectors and promoters. For example, calf chymosin, a serine protease like AChE, and presumably requiring the adoption of tertiary structure conducive to catalytic activity has been successfully expressed in *S. cerevisiae* using YEps and the constitutive PGK promoter. Production reached as much as 5% of total cell protein and the enzyme was shown to have milk clotting activity (Mellor et al, 1983). Human fibrinogen is a complex protein dimer, each monomer of which is composed of three polypeptide chains, alpha, beta, and gamma that are of similar size (67, 57, and 47, KDa respectively) to the polypeptide chain of DACHe. Expression constructs were prepared using the *GAL1* promoter to drive cDNAs of each polypeptide chain. It was found that in *S. cerevisiae* cells expressing all three chains fibrinogen precursors were formed and that under the direction of the  $\alpha$ -factor preprosequence for each chain mature fibrinogen was secreted to the culture medium (Roy et al, 1995). Biological activity of the recombinant fibrinogen was demonstrated by its ability to form a thrombin induced clot in the presence of factor VIIla undergoing gamma chain dimerization and alpha chain polymer formation in order to do so. Thus passage of this complex eukaryotic protein through the secretory pathway of *S. cerevisiae* not only facilitated the processing of each individual chain but also subsequent folding and interaction between the chains to form a final mature and active product. Furthermore, the heavily utilised *GAL1*

promoter succeeded in driving secretion levels to as much as 30mg/L for transformed yeast cells at a density of  $1 \times 10^8$  cells/ml. This compares to  $\approx 100\text{ng DACH/L}$  at a density of  $2 \times 10^6 \approx 5\mu\text{g/L}$  at  $1 \times 10^8$  cells/ml (6000 fold less per cell) reported here. Additionally, the recombinant fibrinogen was not hyperglycosylated and was immunologically indistinct from plasma derived fibrinogen (Roy et al, 1995).

Many other complex eukaryotic proteins including active enzymes have been successfully expressed in *S. cerevisiae* at significantly high levels. Active recombinant human gastric lipase ( $\approx 50$  KDa) with a specific activity comparable to that of the native enzyme has been expressed in *S. cerevisiae* at levels around 11mg/L, 90% of the protein remaining associated with the cell wall (Crabbe et al, 1996). Yields of ovine  $\beta$ -lactoglobulin secreted by virtue of its native signal peptide and indistinguishable in terms of size and structure, immunogenicity, and at the N-terminus (indicating correct processing of the signal peptide) from the native protein purified from sheep milk have been shown to reach 3-4mg/L using the constitutive promoter PGK (Rocha et al, 1996). Other eukaryotic proteins may be expressed at reasonable levels by *S. cerevisiae* but not be processed quite so correctly even with a homologous signal sequence attached. For example active human pancreatic ribonuclease secreted from *S. cerevisiae* at levels of 0.1-0.2 mg/L using the  $\alpha$ -factor preprosequence consists of a heterogeneous population of products that vary not only in the general degree of glycosylation but also in maturation regarding the secretion signal (Ribo et al, 1996).

*S. cerevisiae* then, is capable of processing a relatively complex protein such as DACH through the secretory pathway to a fully functional form that may be indistinguishable from the native protein. Hyperglycosylation may or may not



occur and if this proved to be a problem in terms of activity or for future therapeutic use it may be possible to alleviate it using mutant strains such as *mmn9* (Ballou, 1990). Moir and Dumais (1987) used such a strategy to prevent hyperglycosylation of human  $\alpha$ -1-antitrypsin. Hyperglycosylation does not necessarily prevent efficient secretion or activity, however, as the more recent high level expression (75mg/L using fed batch culture) of biologically active hyperglycosylated human  $\alpha$ -1-antitrypsin by Kang et al (1996) demonstrates.

In the light of a history of successful use the selection of vectors such as pBM150 and pG3 and the promoters *GAL1* and *GPD* for the initial expression of a eukaryotic protein such as AChE in *S. cerevisiae* does not seem unreasonable. The selection of the high copy number vector pG3 and the protease deficient strain BJ2168 as an attempt to increase expression levels following the poor results obtained with the single copy vector pBM150 and the non-protease deficient strain EJL363-6D also seems reasonable. Given that plasmid copy number estimations indicate that the expressed DACH<sub>E</sub> (at least at the levels observed) is not deleterious to cell growth the use of the hormone inducible vector p2UG which utilises the *CYC1* promoter seems unlikely to yield better results in the short term. Similarly, whilst use of a homologous signal sequence such as the  $\alpha$ -factor leader may increase the efficiency of secretion if production is raised substantially and intracellular accumulation becomes a problem (although the native presequence was found to be more efficient for vertebrate AChE expressed in *P. pastoris* (Morel and Massoulie, 1997)) it is unlikely to significantly improve the overall level of expression since the problem with the DACH<sub>E</sub> expression in *S. cerevisiae* seems likely to be centred on a mRNA problem. It should be mentioned that although expression levels of vertebrate AChE in *P. pastoris* were relatively poor, the insect enzyme may be expressed at higher levels

in *Pichia*. The postulated instability of the DACH<sub>E</sub> mRNA in *S. cerevisiae* may not occur in *P. pastoris* due to differences in mRNA processing between the two yeasts. It would be interesting to examine this.

Heterologous expression of a relatively complex eukaryotic protein such as AChE in yeast is often not straightforward and despite the wealth of information available is largely unpredictable. This, by necessity, initially requires an empirical approach which avoids well documented pitfalls. A stable shuttle vector, a strong yeast promoter and trimming of inhibitory leader sequences from the foreign cDNA are all known to be requirements. Plasmid pBM150 and the *GAL1* promoter fulfill these requirements and the leader sequence of the DACH<sub>E</sub> cDNA was almost completely deleted. Plasmid pG3 also confers a yeast transcription terminator thought to aid mRNA stability and BJ2168 is a protease deficient strain of *S. cerevisiae* that should alleviate any degradative problems that may be associated with extraction of a heterologous protein. Although successful expression was achieved using the YEp pG3 and the *GPD* promoter perhaps the complete lack of detectable expression from construct pBM150-DACH<sub>E</sub> indicated that a more radical approach was required such as the use of a *leu-2d* mutant to raise gene copy number as much as 400 fold.

It seems likely that the low level of DACH<sub>E</sub> expression observed in *S. cerevisiae* may be due in large part to an unstable mRNA (6.4). It may be possible to increase expression by replacement or deletion of 5' or 3' sequences of the DACH<sub>E</sub> cDNA that might be responsible for rapid ribonuclease degradation. Further deletion of the DACH<sub>E</sub> 3' UTR upstream of the yeast *PGK* termination sequences is one option. When human placental clotting factor XIII was expressed in *S. cerevisiae* it was found that removal of the 3' UTR resulted in a 50 fold increase in expression (Bishop et al, 1990). Human placental factor XIII is of similar



size and complexity to DACH<sub>E</sub> existing as a native dimer of 166 KDa.

Further deletion or replacement with yeast sequences of the 71 nt 5' UTR remaining of the DACH<sub>E</sub> cDNA or even a portion of the 5' coding sequence may also be warranted if this region is responsible for the postulated transcript instability, although such manipulations are usually performed to alleviate a problem with translation (Kupfer *et al*, 1996). Use of the yeast *GPD* 5' leader and replacement of the coding sequence for the first 30 amino acids of hepatitis B surface antigen with a synthetic DNA sequence composed of preferred yeast codons was found to increase yields by an order of magnitude to  $\approx 3\%$  of total cell protein using a YEp vector and the *GPD* promoter (Bitter and Egan, 1984). Despite the fact that a problem with translation of the DACH<sub>E</sub> is not indicated by the results it might be found that optimization of translation may also beneficially contribute to increased transcript stability.

Expression of the DACH<sub>E</sub> as a fusion protein might also be tried. In this way the 5' end of the transcript may be stabilised and furthermore if a high rate of protein turnover is responsible for the low level production this may also be circumvented. Expression of rat apolipoprotein E (including its native presequence) in *S. cerevisiae* under the *GAL7* promoter was undetectable either in the culture medium or intracellularly until the rat cDNA was fused downstream of the prepro and whole mature sequence of a fungal proteinase (*Mucor pusillus* rennin). The fusion protein was then efficiently secreted to the culture medium at approximately 11mg/L (Nomura *et al*, 1995).

It is possible that there is a problem with transcription despite the fact that the promoters selected have been used successfully for the expression of a wide variety of

foreign proteins. The particular vector sequences in combination with the DACH<sub>E</sub> sequence (including the short segment of 5' UTR remaining in the cDNA) may have diminished promoter strength. Such effects have been observed (Kingsman et al, 1990). Increasing the efficiency of a promoter such as GAL1 by regulated overproduction of its transactivator might be tried (Schultz et al, 1987). Many different hybrid promoters are now available that combine elements from different promoters. These are often more powerful than the promoters from which they were derived. An example is the GRAP1 promoter which combines regions from GAL7 and ADH2 promoters. Used in conjunction with manipulation of the transactivator GAL4 this 'superpromoter' is capable of driving heterologous expression levels to 60% of soluble proteins on galactose induced cells (Hadfield, 1994). Different types of promoter should be tried to assess the potential for increasing the level of DACH<sub>E</sub> expression.

Further characterisation of the *S. cerevisiae* DACH<sub>E</sub> protein is also needed so that detailed comparisons can be made and information gathered concerning processing of the recombinant DACH<sub>E</sub> by *S. cerevisiae*. The results suggest that purification of the enzyme by means of an affinity column containing a specific reversible AChE inhibitor such as procainamide (De La Hoz et al, 1986) or *N*-methylacridinium (Vallette et al, 1983) would be necessary before such studies could be made. Given the level of production from construct pG3-DACH<sub>E</sub> in BJ2168R the purification procedure would be demanding in terms of scale, litres of culture being required to produce even small quantities of purified enzyme. Once a usable quantity of *S. cerevisiae* DACH<sub>E</sub> was available SDS-PAGE analysis could be used to assess the polypeptide composition of the expressed enzyme in terms of size and structure. This is particularly desirable in the light of a possible truncated protein and could not be done here due



to the low levels of expression seen with the pG3-DACHE construct (6.4). Non-denaturing gradient gel electrophoresis would also be desirable to determine the nature of molecular forms. Alternatively one solution to a protein level too small for SDS-PAGE analysis is sedimentation analysis in combination with measurements of activity. This was the technique used by Morel and Massoulie (1997) to gain some insight into the molecular forms of vertebrate AChE produced by *P. pastoris*.

If the *S. cerevisiae* DACHE is actually located in the cell membrane as the results of this study suggest it would be interesting to see whether the enzyme was anchored in the membrane by its hydrophobic C-terminal, by a glycosphospholipid anchor, or even by its secretion signal, although the latter is unlikely. This could be achieved by immunoreactive methods or digestion of the expressed protein with a suitable enzyme such as the PLPC from *Bacillus* spp. used by Fournier et al (1992) in the examination of DACHE from *Xenopus* oocytes. Digested and non-digested forms are then subjected to phase separation in detergent. Digestion of the anchor converts the natural hydrophobic form to a hydrophilic form, activity shifting from detergent to aqueous phases.

The successful expression of an insect AChE in the yeast *S. cerevisiae* is described and the feasibility of its use as a microbial system for the study of insecticide resistant variant AChEs assessed. Despite the advances that have been made in other heterologous systems expression in *S. cerevisiae* offers advantages for mutagenesis studies and the future possibility of safe, efficient, large scale production. Clearly the *S. cerevisiae* system warrants further research into its potential.

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Appendix 1.1. The effect of proteinase K on background interference by *S. cerevisiae* in the Ellman assay (Figure 3.2).

	Mean OD <sub>405</sub>				
Blank	0.062	0.063	0.061	0.058	0.061+/-0.0011
Y+PK+S	0.149	0.097	0.182	0.193	0.155+/-0.0215
Y-PK+S	0.173	0.206	0.164	0.172	0.179+/-0.0093
Y-S	0.166	0.154	0.215	0.181	0.179+/-0.0132
M+PK+S	0.062	0.072	0.063	0.085	0.071+/-0.0053
M-PK+S	0.326	0.285	0.237	0.271	0.280+/-0.0184
M-S	0.067	0.078	0.091	0.068	0.076+/-0.0056

Means +/- S.E.M.

Calculation of *t* values for experimental group comparison where *t* has 6 degrees of freedom.

Group Comparison	<i>t</i>	<i>p</i>
Y+PK+S : Y-PK+S	1.004	>0.1
Y+PK+S : Y-S	0.940	>0.1
Y-PK+S : Y-S	0.016	>>0.1
M+PK+S : M-PK+S	10.915	<0.001
M+PK+S : M-S	0.713	>0.1
M-PK+S : M-S	10.593	<0.001



Appendix 1.2. A comparison between the level of interference caused by two supernatant fractions in the presence and in the absence of substrate (Figure 3.3).

Mean OD<sub>405</sub>

A) SN (3000) + S	0.344	0.588	0.580	0.504+/-0.080
B) SN (3000) - S	0.534	0.622	0.518	0.558+/-0.032
C) SN (13000)+ S	0.456	0.534	0.509	0.500+/-0.023
D) SN (13000)- S	0.493	0.484	0.516	0.498+/-0.010

Means +/- S.E.M.

Calculation of *t* values for experimental group comparison where *t* has 4 degrees of freedom.

Group Comparison	<i>t</i>	<i>p</i>
A : B	0.626	>0.1
C : D	0.0804	>>0.1
B : D	1.780	>0.1

Appendix 1.3. The effect of boiling the supernatant, use of a spun column, and absence of DTNB in the reaction to further investigate the cause of interference (Figure 3.4).

	Mean OD <sub>405</sub>			
A) Sn boiled + S	0.595	0.541	0.607	0.581+/-0.020
B) Sn boiled - S	0.642	0.616	0.661	0.639+/-0.013
C) Sn spun column + S	0.338	0.328	0.324	0.330+/-0.004
D) Sn spun column - S	0.289	0.285	0.282	0.285+/-0.002
E) Sn - DTNB + S	0.073	0.083	0.059	0.071+/-0.006
F) Sn - DTNB - S	0.097	0.082	0.064	0.081+/-0.010

Means +/- S.E.M.

Calculation of *t* values for experimental group comparison where *t* has 4 degrees of freedom

Group Comparison	<i>t</i>	<i>p</i>
A : B	2.432	≈0.1
C : D	9.656	<0.001
E : F	0.791	>0.1
A : C	12.114	<0.001
A : E	23.738	<0.001



Appendix 1.4.a. Levels of interference recorded following enzymatic removal of the cell wall with Zymolyase and subsequent washing of protoplasts (Figure 3.5.a).

Mean OD<sub>405</sub>

A) 1 wash	1.226	1.120	1.217	1.188+/-0.034
B) 2 washes	0.985	1.020	0.980	0.995+/-0.013
C) 4 washes	0.938	0.922	0.923	0.927+/-0.005
D) 8 washes	0.826	0.767	0.740	0.778+/-0.025

Mean +/- S.E.M.

Calculation of *t* values for experimental group comparison where *t* has 4 degrees of freedom

Group Comparison	<i>t</i>	<i>p</i>
A : B	5.324	≈0.01
C : D	5.788	≈0.01
B : C	4.950	≈0.01
A : D	9.674	<0.001

Appendix 1.4.b. Levels of interference recorded following enzymatic removal of the cell wall by Zymolyase with additional digestion by SHP in the presence of DTT and subsequent washing of protoplasts (Figure 3.5.b).

Mean OD<sub>405</sub>

A) 1 wash	1.152	1.083	1.195	1.143+/-0.033
B) 2 washes	1.217	1.140	1.146	1.168+/-0.025
C) 4 washes	0.896	0.869	0.895	0.887+/-0.009
D) 8 washes	0.993	0.861	0.814	0.889+/-0.054

Mean +/- S.E.M.

Calculation of *t* values for experimental group comparison where *t* has 4 degrees of freedom

Group Comparison	<i>t</i>	<i>p</i>
A : B	0.594	>0.1
B : C	10.701	<0.001
C : D	0.049	>>0.1
A : D	4.049	≈0.01

Calculation of *t* value for comparison of Means +/- S.E.M. between Figure 3.5.a. and Figure 3.5.b.

Mean OD<sub>405</sub>

Fig 3.5.a	1.188	0.995	0.927	0.778	0.972+/-0.077
Fig 3.5.b	1.143	1.168	0.887	0.889	1.022+/-0.085

*t* = 0.433 with 4 degrees of freedom

*p* = >>0.1



Appendix 1.5. Product formation above interference levels investigated for induced protoplasts containing pBM150-DAC<sub>h</sub>E assayed using a microtitre plate reader by the method of Ellman et al (1961) (Figure 3.6).

Group	Max Slope mOD <sub>405</sub> /min	nmoles ATCHI hydrolysed /min	Total protein /well
1) Cells transformed with pBM150-DAC <sub>h</sub> E induced on galactose	11.4 23.7 22.4 16.8 19.4 24.6 25.8 19.8 14.7	0.352 0.372 0.369	32.4 29.4 28.5
2) Cells transformed with pBM150-DAC <sub>h</sub> E uninduced	14.7 19.8 22.5 19.8 18.6 24.3 15.9 24.3 20.7	0.349 0.384 0.373	33.3 32.4 28.5
3) Untransformed cells 'induced' on galactose	25.2 29.1 44.4 19.2 26.7 30.0 28.5 30.3 31.5	0.605 0.465 0.553	37.8 33.0 39.3
4) Untransformed cells 'uninduced'	28.8 30.3 31.2 32.7 31.2 33.0 28.5 27.3 37.2	0.553 0.594 0.570	43.2 40.5 40.5
5) Mosquito homogenate control	351.0 351.0 386.1 342.3 398.1 374.7 348.9 335.4 375.3	6.667 6.832 6.493	Not Sampled

Appendix 1.6. Transformed and untransformed cells assayed by the method of Ellman compared in terms of specific activity (Figure 3.7).

Group	Units of AChE Activity/mg Protein	Mean Units of Activity +/- S.E.M.
1	10.86 12.65 12.95	12.15 +/- 0.653
2	10.48 11.85 13.09	11.81 +/- 0.756
3	16.00 14.09 14.07	14.72 +/- 0.641
4	12.80 14.67 14.07	13.85 +/- 0.552

*t* values for comparison of induced transformed cells (1) with control groups (2-4) where *t* has 4 degrees of freedom

Group comparison	<i>t</i>	<i>p</i>
1 : 2	1.04	>0.1
1 : 3	2.81	≈0.05
1 : 4	1.99	>0.1



Appendix 2.1. Activity of transformed and untransformed *S. cerevisiae* cell extracts (glass bead protocol) from exponential and stationary phase cultures (Sabine method) (Figure 5.1).

OD <sub>600</sub> 0.1 Supernatant	nmoles ACHI hydrolysed/min*	Mean nmoles ACHI hydrolysed/min +/- S.E.M.
BJ2168R	15.0 15.0 18.3	16.1 +/- 1.10
BJ2168W	3.3 3.3 6.7	4.4 +/- 1.14
BJ2168	3.3 3.3 8.3	5.0 +/- 1.67

OD <sub>600</sub> 0.1 Total Cell Extract	nmoles ACHI hydrolysed/min	Mean nmoles ACHI hydrolysed/min +/- S.E.M.
BJ2168R	71.7 71.7 73.3	72.2 +/- 0.53
BJ2168W	8.3 6.7 3.3	6.1 +/- 1.48
BJ2168	6.7 8.3 8.3	7.8 +/- 0.53

OD <sub>600</sub> 0.45 Total Cell Extract	nmoles ACHI hydrolysed/min	Mean nmoles ACHI hydrolysed/min +/- S.E.M.
BJ2168R	86.7 90.0 85.0	87.2 +/- 1.50
BJ2168W	11.7 13.3 13.3	12.8 +/- 0.53
BJ2168	20.0 13.3 13.3	15.5 +/- 2.24

*t* values for group comparison where *t* has 4 degrees of freedom:

Group comparison OD <sub>600</sub> Supernatant	<i>t</i>	<i>p</i>
BJ2168R : BJ2168W	7.41	≈0.002
BJ2168R : BJ2168	5.56	≈0.005
BJ2168W : BJ2168	0.30	>>0.1

Group comparison OD <sub>600</sub> 0.1 Total Cell Extract	<i>t</i>	<i>p</i>
BJ2168R : BJ2168W	42.16	<<0.001
BJ2168R : BJ2168	85.30	<<<0.001
BJ2168W : BJ2168	1.08	>0.1

Group comparison OD <sub>600</sub> 0.45 Total Cell Extract	<i>t</i>	<i>p</i>
BJ2168R : BJ2168W	47.63	<<0.001
BJ2168R : BJ2168	26.83	<0.001
BJ2168W : BJ2168	1.18	>0.1



Appendix 2.2. Quantification of activity levels of DAcHE extracted from *S. cerevisiae* protoplasts (Sabine method) (Figure 5.2).

	nmoles ACHI hydrolysed/min*	Total Protein in Assay (µg)	Units of AChE Activity/mg Protein	Mean Units of AChE Activity +/- S.E.M.
BJ2168R	50.0	663.0	75.47	70.89 +/- 3.39
	45.0	700.0	64.29	
	48.3	663.0	72.91	
BJ2168W	3.3	512.5	6.49	3.09 +/- 1.88
	0.0	612.5	0.00	
	1.66	600.0	2.77	
BJ2168	3.3	512.5	6.50	3.13 +/- 1.88
	0.0	662.5	0.00	
	1.66	575.0	2.89	

\* nmoles of ACHI hydrolysed/min are calculated from the absorbance at 540nm recorded after 30 min and compared to the absorbance recorded for a mean standard (4mM ACHI) (2.22.5). OD Units are converted to absolute units by a standard curve of ACHI concentration against OD<sub>540</sub>.

t values with 4 degrees of freedom:

Comparison	t	p
BJ2168R : BJ2168W	17.17	<0.001
BJ2168R : BJ2168	15.57	<0.001
BJ2168W : BJ2168	0.05	>>0.1

Appendix 2.3. Quantification of activity levels of DACH<sub>E</sub> extracted from *S. cerevisiae* protoplasts (Ellman method). Controls show levels of activity derived mainly from interference (Figure 5.5).

	nmoles ATCHI hydrolysed /min**	Total Protein/ well (µg)	Units of AChE Activity/ mg Protein	Mean Units of AChE Activity+/ - S.E.M.
BJ216R	0.800	22.2	36.04	27.32 +/- 4.49
	0.625	25.2	24.80	
	0.450	21.3	21.13	
BJ2168W	0.175	25.2	6.94	8.14 +/- 0.61
	0.225	25.2	8.90	
	0.175	20.4	8.58	
BJ2168	0.075	16.2	4.63	7.83 +/- 2.80
	0.225	16.8	13.39	
	0.100	18.3	5.46	

\*\* Since the extinction co-efficient of the yellow anion is known (Ellman et al, 1961) rates of change in absorbance can be converted to absolute units:

$$\text{Rate (moles/l/min)} = \text{Change in OD per min}/1.36 \times 10^4$$

Each well of the microtitre plate contained a total assay volume of 250µl.

*t* values with 4 degrees of freedom:

Comparison	<i>t</i>	<i>p</i>
BJ2168R : BJ2168W	4.24	≈0.01
BJ2168R : BJ2168	3.69	≈0.02
BJ2168W : BJ2168	0.33	>>0.1



Appendix 2.4. The effect of the insecticide Bendiocarb on the activity of DACH<sub>E</sub> from BJ2168R compared to controls (Figure 5.7).

	Activity in mOD Units /min Uninhibited	Activity in mOD Units/min Inhibited	Activity as % of Uninhibited	Mean Activity as % of Uninhibited +/- S.E.M.
BJ2168R	47.4 46.8 38.1 30.0 38.4 34.5 24.6 24.6 28.2	22.2 22.5 18.0 19.2 24.5 18.9 7.2 13.8 13.8	46.8 48.1 47.2 64.0 63.8 54.8 29.2 56.1 8.9	50.9 +/- 3.52
BJ2168W	11.4 4.2 14.4 15.3 14.1 7.5 13.2 5.1 13.2	7.5 10.2 12.9 14.5 12.4 6.6 11.1 9.6 9.6	65.8 >100 89.6 94.8 87.9 88.0 84.1 >100 72.2	86.9 +/- 3.88
BJ2168	6.3 3.9 5.4 26.1 5.1 5.7 4.2 9.3 6.6	3.1 6.1 3.4 8.5 4.5 4.3 2.3 8.2 7.9	49.2 >100 63.0 32.6 88.2 75.4 54.8 88.2 >100	72.4 +/- 7.90

t values with 16 degrees of freedom:

Comparison	t	p
BJ2168 : BJ2168W	1.65	>0.1
BJ2168 : BJ2168R	2.50	≈0.02
BJ2168W : BJ2168R	6.87	<0.001

Appendix 3.1. Plasmid copy number estimations.

Densitometry tracings of the bands on the autoradiograph were used to calculate the number of plasmids per cell in relation to the number of copies of ribosomal RNA genes using the formula:

$$140 \times \frac{\text{Area under densitometry peak for band 4}}{\text{Area under densitometry peak for band 1}} \times \frac{2.6}{4.9}$$

Where band 4 is a ribosomal band and band 1 is the DAcHE band with some pUC18 attached. 2.6 and 4.9 are the lengths of homology between the probe and the target band for ribosomal and pUC18 DNA respectively.

BJ2168R		BJ2168W	
rDNA	Plasmid	rDNA	Plasmid
3.25	2.65	3.76	2.86
1.97	1.69	2.22	2.08
3.86	2.21	4.75	2.82

rDNA = 140 copies per genome.

$$140 \times \text{Plasmid/rDNA} \times \frac{2.6}{4.9}$$

BJ2168R				
74.28	X	0.815	=	60.54
		0.858	=	63.73
		0.573	=	42.56
			=	55.61+/- 11.43

BJ2168W				
74.28	X	0.761	=	56.53
		0.937	=	69.60
		0.594	=	44.10
			=	56.74+/- 12.75

t = 0.114 with 4 degrees of freedom  
p = >>0.1



Appendix 3.2. The effect of different concentrations of PMSF on the expressed DACE and *Torpedo* AChE (Figure 6.6).

	BJ2168R	BJ2166W	<i>Torpedo</i>
Activity mOD/min Uninhibited	137.4 121.5 123.3	54.8 53.7 47.9	234.4 289.2 318.9
Activity mOD/min Inhibited 1mM PMSF	140.9 127.8 105.6	56.6 51.7 53.6	294.0 272.1 230.7
Activity as % Uninhibited  Mean +/- S.E.M.	102.5 105.2 85.7 97.8 +/- 6.12	103.3 96.3 111.9 103.8 +/- 4.52	125.4 94.1 72.5 97.3 +/- 15.39
Activity mOD/min Inhibited 2mM PMSF	128.6 133.2 105.6	52.4 55.7 41.0	199.3 202.5 201.0
Activity as % Uninhibited  Mean +/- S.E.M.	93.6 109.6 85.7 96.3 +/- 7.06	95.6 103.7 85.6 95.0 +/- 5.24	85.8 70.0 63.0 72.7 +/- 6.50
Activity mOD/min Inhibited 4mM PMSF	92.4 103.1 68.8	38.3 68.2 46.0	183.2 155.2 169.2
Activity as % Uninhibited  Mean +/- S.E.M.	67.3 84.9 55.8 69.3 +/- 8.46	69.9 127.0 96.0 97.6 +/- 16.52	78.2 53.7 53.6 61.8 +/- 8.19

*t* values for group comparison where *t* has 4 degrees of freedom:

Group Comparison 1mM PMSF	<i>t</i>	<i>p</i>
BJ2168R : BJ2168W	0.79	>0.1
BJ2168R : <i>Torpedo</i>	0.03	>>>0.1
BJ2168W : <i>Torpedo</i>	0.41	>0.1

Group Comparison 2mM PMSF	<i>t</i>	<i>p</i>
BJ2168R : BJ2168W	0.15	>>0.1
BJ2168R : <i>Torpedo</i>	2.42	≈0.075
BJ2168W : <i>Torpedo</i>	2.61	≈0.05

Group Comparison 4mM PMSF	<i>t</i>	<i>p</i>
BJ2168R : BJ2168W	1.61	≈0.1
BJ2168R : <i>Torpedo</i>	0.73	>0.1
BJ2168W : <i>Torpedo</i>	1.94	≈0.1



Appendix 3.3. Quantification of activity levels of uninhibited DAcHE from BJ2168R (PMSF inhibition study).

	nmoles ATCHI hydrolysed /min	Total Protein/ well ( $\mu$ g)	Units of AChE Activity /mg Protein	Mean Units of AChE Activity +/- S.E.M.
BJ2168R	2.525	37.2	67.88	60.51 +/- 3.83
	2.225	40.4	55.07	
	2.250	38.4	58.59	
BJ2168W	1.050	75.6	13.89	14.86 +/- 0.71
	0.975	60.0	16.25	
	0.875	60.6	14.44	
<i>Torpedo</i>	4.309	39.6	108.81	114.46 +/- 14.17
	5.316	57.0	93.26	
	5.850	41.4	141.30	

#### Appendix 3.4. Using activity to estimate enzyme concentration.

$K_{cat}$  = N° of molecules of substrate transformed to product per molecule of enzyme per sec.

The assumption was made that the protein complement of BJ2168W was equal to that of BJ2168R and equivalent aliquots of BJ2168W protoplast extracts were added to microtitre plate wells containing *Torpedo* AChE.

DACHE from BJ2168R was inactivated by 30.7% at 4mM PMSF.

AChE from *Torpedo* was inactivated by 38.2% at 4mM PMSF.

$K_{cat}$  of recombinant DACHE =  $K_{cat}$  *Torpedo* AChE X 30.7/38.2

= 8000 (Taylor et al, 1974) X 0.803665 = 6429.3

N° of moles of enzyme =  $x/K_{cat} \cdot y$

Mean number of nmoles ATCHI hydrolysed/min by BJ2168R DACHE  
= 2.33 +/- 0.10 - 0.501 (Correction factor for cell wall interference (Appendix 6.5)) = 1.829 nmoles/min = 0.0305 nmoles/sec = 24.3866 in 800 sec (40 cycles/interval 20 sec)

N° of nmoles of recombinant DACHE = 24.3866/6429.3 X 800

= 4.74 X 10<sup>-6</sup> nmoles DACHE/well

Mean protein/well = 38.6 +/- 0.93 µg

DACHE monomer = 73 KDa (Fournier et al, 1988)

1 nmole of DACHE = 73000ng

4.74 X 10<sup>-6</sup> nmoles = 0.346ng DACHE

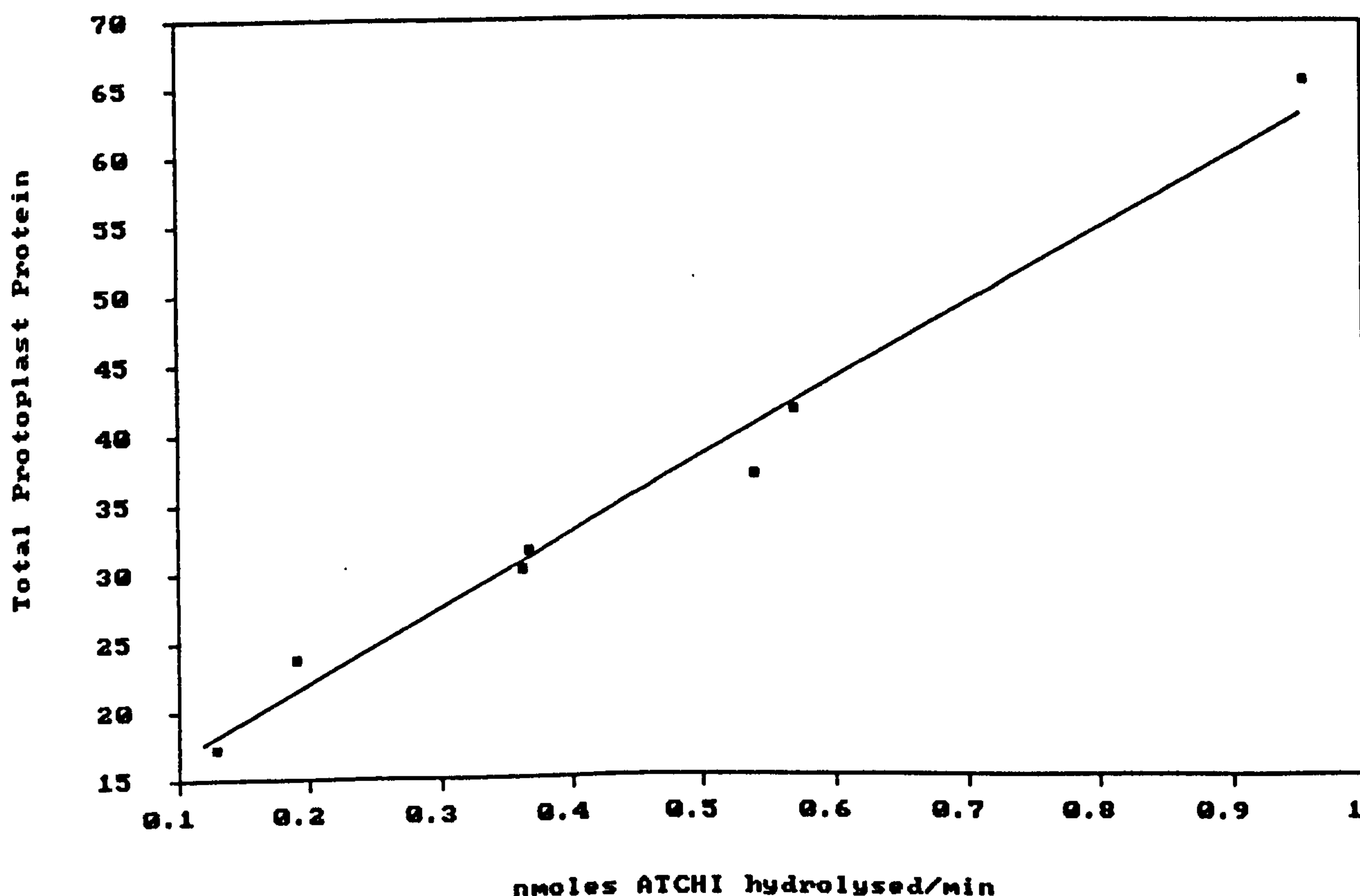
Mean total protoplast protein/well = 38600ng

0.0009% of total BJ2168R protoplast protein is active DACHE.



### Appendix 3.5. Relationship Between Cell Wall Interference and Total Protoplast Protein.

If the data from *S. cerevisiae* protoplast extracts from microtitre plate assays using the Ellman method are collated a relationship emerges between the level of cell wall interference encountered and the amount of total protoplast protein. It can be seen that above a threshold of around 10 $\mu$ g total protoplast protein there is an approximate increase in cell wall interference of 0.01 OD<sub>405</sub> Units/min for an increase of 10 $\mu$ g total protoplast protein (below a threshold of 10 $\mu$ g the level of interference approximates to that of the blank or lower ( $\approx$  0.002-0.003 OD<sub>405</sub> Units/min) (Appendices 1.5, 2.3, 2.4, 3.2, 3.3). Conversion of Units of absorbance to mean nmoles of ATCHI hydrolysed/min for *S. cerevisiae* protoplast extracts when plotted against total protoplast protein gave the following relationship:



Mean nmoles ATCHI Hydrolysed/min +/- S.E.M.	Mean Total Protoplast Protein ( $\mu$ g) +/- S.E.M.
0.130 +/- 0.05	17.1 +/- 0.63
0.191 +/- 0.02	23.6 +/- 1.60
0.364 +/- 0.006	30.1 +/- 1.18
0.369 +/- 0.01	31.4 +/- 1.47
0.541 +/- 0.04	36.7 +/- 1.90
0.572 +/- 0.01	41.4 +/- 0.90
0.959 +/- 0.05	65.4 +/- 5.12

The data from which the means were calculated are given in Appendices 1.5, 2.3, and 3.3.

The straight line relationship can be used to approximate a 'correction factor' for cell wall interference in calculating recombinant DACH<sub>E</sub> activity levels from BJ2168R based upon mean total protein per well.



Appendix 3.6.a. Activity levels associated with the cell membrane fraction of BJ2168R protoplasts compared to levels recorded for whole protoplast extracts (Figure 6.9.a).

	nmoles ACHI hydrolysed /min	Mean nmoles ACHI hydrolysed/ min +/- S.E.M.	Total Protein in Assay (µg)	Total Protein Before Assay (µg)
BJ2168R	25.0	21.67+/-	96.9	525.0
Memb Fract	18.33	3.35	90.6	543.8
BJ2168R	31.67	30.00+/-	500.0	-
Whole Ext	28.33	1.68	484.4	-
BJ2168W	3.33	2.500+/-	565.6	-
Whole Ext	1.67	0.83	534.4	-

Appendix 3.6.b. The mean specific activity associated with the cell membrane extract of BJ2168R protoplasts compared to that calculated for whole protoplast extracts (Figure 6.9.b).

	nmoles ACHI hydrolysed /min	Total Protein in Assay	Units of DACHe Activity /mg Protein	Mean Units of DACHe Activity +/- S.E.M.
BJ2168R	25.00	96.9	258.53	230.43+/-
Memb Fract	18.33	90.6	202.32	28.19
BJ2168R	31.67	500.0	63.34	60.92+/-
Whole Ext	28.33	484.4	58.49	2.43
BJ2168W	3.33	565.6	5.89	4.51+/-
Whole Ext	1.67	534.4	3.12	1.39

*t* values for comparison of specific activities (Appendix 3.6.b) where *t* has 2 degrees of freedom:

Group comparison	<i>t</i>	<i>p</i>
BJ2168R Memb F: BJ2168R Whole E	6.01	≈0.02
BJ2168R Memb F: BJ2168W Whole E	8.03	≈0.01
BJ2168R Whole E: BJ2168W Whole E	20.19	≈0.002



Appendix 3.7. Activity levels of BJ2168R protoplasts stabilized in 1M sorbitol compared to levels typically associated with lysed protoplast extracts (Figure 6.11).

	nmoles ACHI hydrolysed/min*	Mean nmoles ACHI hydrolysed/min +/- S.E.M.
BJ2168R Lysed	46.67 61.67	54.17+/- 7.52
BJ2168R Stabil.	11.67 8.33	10.00+/-1.68
BJ2168W Lysed	6.67 6.67	6.67+/-0.00
BJ2168W Stabil.	>Std >Std	-

\* nmoles ACHI hydrolysed/min calculated as detailed in Appendix 2.2.

>Std = an absorbance reading greater than the Standard.

t values for group comparison where t has 2 degrees of freedom:

Group Comparison	t	p
BJ2168R Lysed: BJ2168R Stabil.	5.75	≈0.03
BJ2168R Lysed: BJ2168W Lysed	6.33	≈0.02
BJ2168W Lysed: BJ2168R Stabil.	1.99	>0.1

Appendix 3.8. Activity levels of the supernatant fraction of protoplast preparations compared to lysed protoplast extracts (Figure 6.12).

	nmoles ACHI hydrolysed/min*	Mean nmoles ACHI hydrolysed/min +/- S.E.M.
BJ2168R Protoplast Supernatant (A)	1.67 >Std 1.67	1.67 +/- 0.00
BJ2168R Lysed Protoplasts (B)	23.33 25.00 25.00	24.44 +/- 0.56
BJ2168W Protoplast Supernatant (C)	8.33 1.67 >Std	5.00 +/- 3.36
BJ2168W Lysed Protoplasts (D)	3.83 1.67 3.33	2.94 +/- 0.65

\* nmoles ACHI hydrolysed/min calculated as detailed in Appendix 5.2.

>Std = An absorbance reading greater than the Standard.

*t* values for group comparison where *t* has 2, 3 or 4 degrees of freedom:

Group Comparison	<i>t</i>	<i>p</i>
A : B	31.67	<0.001
A : C	0.99	>0.1
A : D	1.51	>0.1
B : C	7.52	≈0.01
B : D	25.05	<0.001
C : D	0.76	>0.1



Appendix 3.9. Comparison of protoplasting methods (levels of DACHe activity obtained) (Sabine method) (Figure 6.13).

	nmoles ACHI hydrolysed /min*	Total Protein in Assay (µg)	Units of DACHe Activity/ mg Protein	Mean Units of DACHe Activity +/- S.E.M.
BJ2168R	40.00	762.5	52.46	55.32 +/- 3.30
37°C	40.00	775.0	51.61	
30 min (A)	43.33	700.0	61.90	
BJ2168R	31.67	637.5	49.68	50.19 +/- 0.67
RT°C	28.33	550.0	51.51	
15 min (B)	33.33	675.0	49.38	
BJ2168W	5.00	787.5	6.35	5.29 +/- 0.59
37°C	3.33	775.0	4.30	
30 min (C)	3.33	637.5	5.22	
BJ2168W	5.00	887.5	5.63	6.07 +/- 0.48
RT°C	5.00	900.0	5.56	
15 min (D)	6.67	950.0	7.02	

\* nmoles of ACHI hydrolysed/min calculated as detailed in Appendix 2.2.

t values for group comparison where t has 4 degrees of freedom:

Group comparison	t	p
A : B	1.53	>0.1
A : C	14.93	<0.001
A : D	14.78	<0.001
B : C	50.36	<<0.001
B : D	53.90	<<0.001
C : D	1.03	>0.1

Appendix 3.10. Calculation of Specific Activity for *PDA1* and DACE cDNA Radiolabelled Probes.

The proportion of  $^{32}\text{P}$  labelled  $\alpha$ -dCTP that had been incorporated into each probe was based on differential precipitation with TCA followed by Cerenkov counting of dry filters in the  $^3\text{H}$  channel of a liquid scintillation counter and calculated in dpm/ $\mu\text{g}$  as suggested by the Amersham protocol as follows:

cpm in washed filter/cpm in unwashed filter = proportion incorporated

DACE cDNA:  $1077233/1947375 = 55\%$   
*PDA1*:  $703643/2465943 = 29\%$

Total Mass of DNA (template + probe) =

$[\mu\text{Ci added}] [13.2] [\% \text{ incorporation}]/\text{specific activity of } ^{32}\text{P dCTP} + \text{starting template(ng)}$

For DACE cDNA =  $50 \times 13.2 \times 55/3000 + 25 = 37.1\text{ng}$   
For *PDA1* =  $50 \times 13.2 \times 29/3000 + 25 = 31.38\text{ng}$

Radioactivity incorporated during labelling reaction in dpm:  $[50\mu\text{Ci}][2.2 \times 10^4][\% \text{ incorporation}]$

For DACE cDNA =  $50 \times (2.2 \times 10^4) \times 55 = 6.1 \times 10^7 \text{ dpm}$   
For *PDA1* =  $50 \times (2.2 \times 10^4) \times 29 = 3.2 \times 10^7 \text{ dpm}$

Specific Activity in dpm/ $\mu\text{g}$ :

For DACE cDNA =  $[6.1 \times 10^7][10^3]/37.1 = 1.64 \times 10^9 \text{ dpm}/\mu\text{g}$   
For *PDA1* =  $[3.2 \times 10^7][10^3]/31.38 = 1.02 \times 10^9 \text{ dpm}/\mu\text{g}$

dpm = disintegrations per minute

$1 \mu\text{Ci} = 2.2 \times 10^6 \text{ dpm}$